

**AN INVESTIGATION INTO  
TETRASPANIN CD151  
AS NOVEL PROGNOSTIC MARKERS IN  
POOR OUTCOME ENDOMETRIAL CANCER**

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## **ABSTRACT**

### **Background:**

Type II endometrial carcinoma, sarcoma and carcinosarcoma account for 10% of uterine malignancies but 50% of recurrences. Survival at recurrence is poor and better prognostic markers are needed to guide therapy. The prognostic significance of the novel markers clusterin and tetraspanin CD151 were evaluated in a cohort of poor outcome endometrial malignancies, along with oestrogen receptor, progesterone receptor, p53 and human epidermal growth factor receptor 2. Immunohistochemistry profiles and survival outcome between grade 3 endometrioid cancers and type 2 cancers were compared.

### **Material and Methods:**

Tissue microarrays constructed from 156 poor outcome uterine malignancies, tested with immunohistochemistry and staining were correlated with clinicopathological, mortality and survival data.

### **Results:**

Expression of CD151 was significantly higher in uterine papillary serous and clear cell carcinoma (USPC+CC) compared to grade 3 endometrioid carcinoma, sarcoma or carcinosarcoma. All other markers were not prognostic for survival. Except for CD151, there was no significant difference in marker positivity, age, stage or survival between G3 EEC and UPSC+CC.

### **Conclusion:**

CD151 is a novel marker in type 2 cancers that may guide therapeutic decisions. These data also suggest that grade 3 EEC is better characterised as a type II endometrial cancer and may benefit from similar treatment.

## **DEDICATION**

I dedicate this thesis to my father with admiration and endless love.

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## PAPERS PUBLISHED FROM THIS THESIS

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Gynecol Oncol. 2012 Jan;124(1):15-20. doi: 10.1016/j.ygyno.2011.07.030. Epub 2011 Aug 23.  
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# List of Abbreviations

TAH	Total abdominal hysterectomy
TLH	Total laparoscopic hysterectomy
BSO	Bilateral salpingo-oophorectomy
ER	Progesterone receptor
PR	Oestrogen receptor
RT	Radiotherapy
CT	Chemotherapy
WHO	World Health Organisation
FIGO	International Federation of Gynecology and Obstetrics
TNM	TNM Classification of Malignant Tumours (T=Tumour, N=Nodes, M=Metastases)
CR	Complete Regression
PR	Partial Regression
SD	Stable Disease
PD	Progressive Disease
GHNHSFT	Gloucestershire Hospitals National Health System Foundation Trust
NHS	National Health System
H&E	Haematoxylin & Eosin staining method
TBS	Tris-Buffered Saline
IMS	Industrial Methylated Spirit
DSS	Disease Specific Survival
PFS	Progression Free Survival
EBRT	External Beam Radiation Therapy
VBT	Vaginal Brachytherapy

Gy	Gray - SI unit for radiation
GP	General Practitioner
TMA	Tissue microarray
pTEN	Phosphatase and tensin homolog
P16	Cyclin-dependent kinase inhibitor 2A, (CDKN2A)
P53	Protein 53 or tumour protein 53
K-ras	Kras protein is a GTPase encoded by the KRAS gene
Her2	HER2 (Human Epidermal Growth Factor Receptor 2) is a member of the epidermal growth factor receptor (EGFR/ErbB) family also known as Neu, ErbB-2, CD340 or p185 is a protein encoded by the <i>ERBB2</i> gene.
Erb B2	See Her2
PIK3CA	Phosphatidylinositol 3'-kinase catalytic subunit. Synonyms: PI3-kinase subunit alpha, PI3K, PI3K-alpha
MSI	Microsatellite Instability
β-catenin	Beta-catenin is a protein encoded by the CTNNB1 gene
E-Cadherin	To (E) epithelial tissue bound member of a sub-group of cadherins called 'classical'. Composed of "calcium-dependent adhesion", cadherins are a class of type-1 transmembrane proteins.
PPP 2R1A	Subunit of protein phosphatase 2
ARID1A	“AT-rich interactive domain-containing protein 1A” is a protein that is encoded by the <i>ARID1A</i> gene. As a member of the SWI/SNF family, it has helicase and AT-Pase activities.
LVSI	LymphoVascular Space Invasion

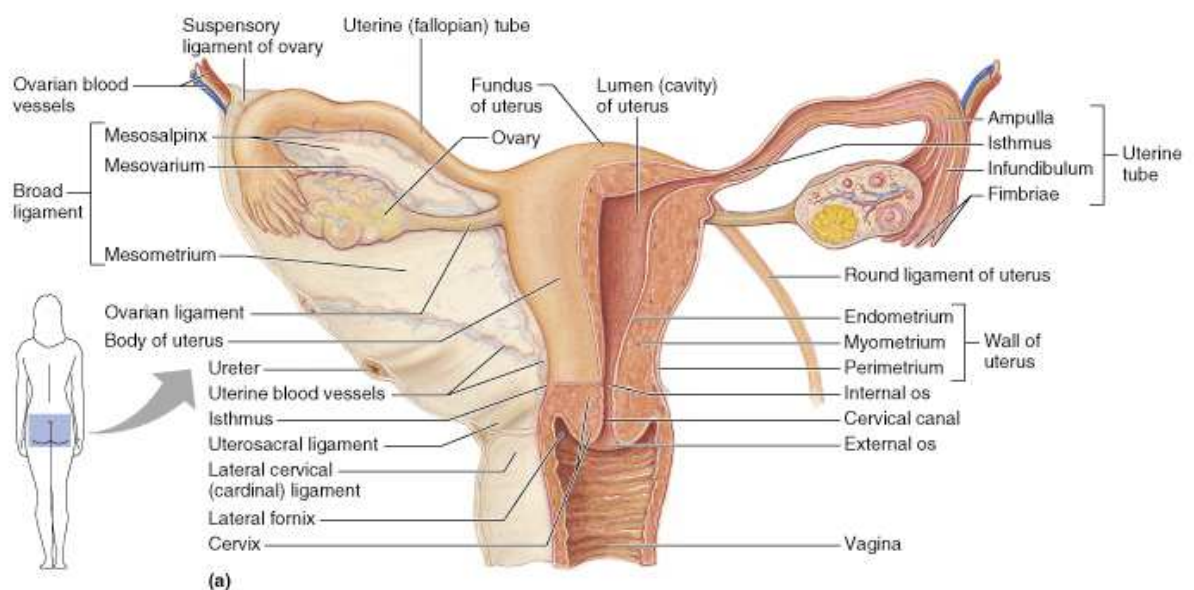
# **CHAPTER**

## **1 INTRODUCTION**

## 1.1 Anatomy and histology of the uterus

The uterus is a pear-shaped organ consisting of a fundus, body, isthmus and cervix. The fallopian tubes enter at each superolateral angle, above which lies the uterine fundus. The uterine body is flexed on the cervix (anteflexion) while the whole uterus is tipped forward (anteversion). The uterus measures about 7.5 cm in length, 5 cm in breadth at its fundal end, and nearly 2.5 cm in thickness; it weighs between 30 to 40 g.

**Figure 1.1**



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### **Anatomy of the uterus and adnexae.**

The uterus is divided into body (upper two-thirds) and cervix. The cervical canal traverses the internal os and emerges as the external os at the vaginal vault. The walls of

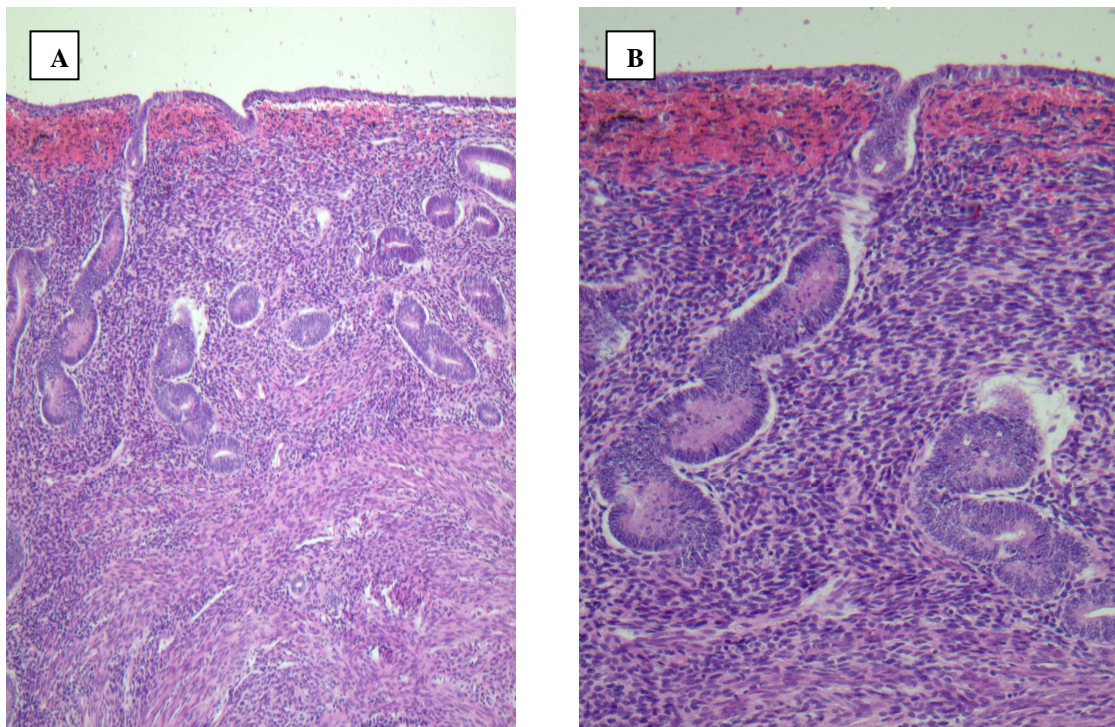


the uterus are composed of a mucosal layer (mucosa), the endometrium, and a fibromuscular layer, the myometrium (muscularis). The peritoneal surface of the uterus is covered by serosa. The myometrium is formed by layers of muscle fibres. The muscular tissue hypertrophies during pregnancy, and GAP-junctions between cells become more frequent.

The endometrium consists of a simple columnar epithelium (ciliated cells and secretory cells) resting on a layer of connective tissue stroma. The endometrial mucosa is invaginated to form many simple tubular uterine glands. The glands extend through the entire thickness of the stroma. (Figure 1.2, see page 4) The stromal cells of the endometrium are embedded in a network of reticular fibres. In women of reproductive age, two layers of endometrium can be distinguished: The outer functional layer (functionalis) is the luminal part of the endometrium. This layer is built up following menstruation in the proliferative, first half of the menstrual cycle induced by oestrogen (follicular phase of menstrual cycle). Later changes in this layer are induced by luteal progesterone (luteal phase) providing an optimal environment for the nidation and growth of the embryo. This layer is shed during menstruation.

The basal layer (basalis) is attached to the myometrium and below the functional layer. It is not shed during the menstrual cycle and forms the basis from which the functional layer redevelops at the beginning of every menstrual cycle.

**Figure 1.2**



**Normal uterine wall, hematoxylin & eosin staining: A. x200, with myometrium shown in lower half of the picture; B. x400, with invaginated gland extending through the thickness of the endometrial stroma.**

## 1.2 Uterine malignancies – overview

Uterine malignancies arise from the body of the uterus and can be broadly grouped into:

- Endometrial carcinoma (90-95%)
- Sarcoma of the uterus (3-7%)
- Carcinosarcoma of the uterus (1%)

The term endometrial cancer is often used to address every form of malignancy of the corpus uteri. However, it is actually a histopathological term exclusively referring to malignant tumours arising from the endometrium. Sarcomas are separately classified and staged. Carcinosarcoma or Malignant Mixed Mullerian Tumour (MMMT) used to be included in studies of uterine sarcoma and is classified as such by the World Health Organisation (WHO).<sup>1</sup> However, carcinosarcomas are now believed to originate from a monoclonal carcinoma cell, and have recently been classified as metaplastic or dedifferentiated form of endometrial carcinoma. This has now been recognised by FIGO (International Federation of Gynecology and Obstetrics) and the current FIGO staging 2009 of endometrial cancer includes carcinosarcoma.<sup>2 3</sup>

By convention, published literature on the epidemiology and survival from uterine cancer pertains to endometrial carcinoma only and does not include sarcoma and carcinosarcoma. In this study endometrial carcinoma, sarcoma and carcinosarcoma were included, thus their respective diagnosis, management and prognosis will be outlined in separate sections.

## **1.3 Endometrial carcinoma**

### **1.3.1 Epidemiology**

While the incidence and mortality rates from several other cancers have plateaued or decreased in the last decade the incidence of endometrial cancer has been rising throughout Europe and it is currently the most common type of gynaecological cancer.<sup>4</sup>

Worldwide it is the seventh most common cancer of women, with 189,000 new cases and 45,000 deaths each year and with the highest incidence rates in the Western world countries.<sup>5 6</sup>

In the United Kingdom endometrial cancer is currently the fourth most common cancer in women. A rapid increase by 40% has been observed since 1993, to an incidence of 7835 in 2009 and 1937 deaths in 2010.<sup>7</sup>

The age standardised incidence rate of endometrial cancer in the UK was 19.4 in 2008 which compares to 16.2 in cancer of the ovary, 8.7 in cancer of the cervix and 2.4 in cancer of the vulva. In the majority (93%) of cases endometrial cancer is diagnosed in women aged over 50 years with only few women diagnosed under the age of 35. Incidence rises rapidly from just under 40 cases per 100,000 at the age of 50 to a peak of 83 per 100,000 females in their early seventies. Uterine cancer incidence rates decline after the age of 75.

In the period 2005-2009, 77% of women in England survived their uterine cancer for five years or more. However, an increase in mortality has been observed. In the period between 1998 and 2010 the mortality rate of uterine cancers in the UK has risen by almost a fifth (17.9%) from 3.1 in 1997-1999 to 3.7 per 100,000 people in 2008-2010.<sup>8</sup>

Furthermore, data from the United Kingdom West Midlands registry confirm more specifically that mortality in some groups of endometrial cancer has worsened.<sup>4</sup> Findings within the United States based Surveillance, Epidemiology and End Results database (SEER) study of more than 45,000 women with endometrial cancer, suggest that the increase in mortality may be related to an increased rate of advanced-stage cancers and high-risk histologies namely uterine papillary serous carcinoma (UPSC) and clear cell carcinoma.<sup>9 10</sup> It is believed that the main causes for the observed increase in incidence and mortality in endometrial cancer are an increase in life expectancy, more women being overweight, having fewer or no children and tamoxifen use.<sup>4 11 12</sup>

### **1.3.2 Classification**

The most common endometrial cancer cell type is endometrioid adenocarcinoma (75-80%), which is composed of malignant glandular epithelial elements. Sometimes an admixture of squamous metaplasia is also found. Adenosquamous tumours contain malignant elements of both glandular and squamous epithelium. Uterine papillary serous (UPSC) and clear cell (CC) carcinomas and tumour of mixed epithelial histology comprise approximately 14% of endometrial cancers. Mucinous, squamous and undifferentiated tumours are rarely encountered. The WHO classification and respective frequency of endometrial carcinoma cell types are outlined on the next page (Table 1.1, see page 8).<sup>13</sup>

**Table 1.1.**

	<b>TYPES OF ENDOMETRIAL CARCINOMA</b>
<b>1</b>	<b>Endometrioid (75%–80%)</b>
	<b>a. Ciliated adenocarcinoma.</b>
	<b>b. Secretory adenocarcinoma.</b>
	<b>c. Papillary or villo-glandular.</b>
	<b>d. Adenocarcinoma with squamous differentiation.</b>
<b>2</b>	<b>Uterine papillary serous (&lt;10%).</b>
<b>3</b>	<b>Mucinous (1%).</b>
<b>4</b>	<b>Clear cell (4%).</b>
<b>5</b>	<b>Squamous cell (&lt;1%).</b>
<b>6</b>	<b>Mixed (10%).</b>
<b>7</b>	<b>Undifferentiated.</b>

**WHO classification and respective frequency of endometrial carcinomas.**

### **1.3.3 Endometrial carcinoma and the dualistic model of endometrial tumourigenesis**

Apart from the FIGO classification which divides endometrial carcinoma solely into different histological types as described above, current concepts of endometrial cancer integrate traditional histopathology with pathogenetic mechanisms and the dividing line is the clinically relevant recognition of clinical outcome and prognosis.

Endometrial cancer comprises not only the majority of tumours that respond well to treatment and confer a good prognosis but also a significant group of less common cancers that can be very aggressive, and account for a greatly disproportionate amount of deaths from uterine cancers. In 1983 Bokhman recognised this dilemma and postulated a classification of endometrial carcinomas into two types (Table 1.2, see page 10). Although this classification is an oversimplification, it is currently a widely accepted concept.<sup>14</sup>

Type I endometrial carcinoma, is oestrogen dependent, of low grade endometrioid histology, biologically indolent and carries a good prognosis.

Type II endometrial cancers are non-oestrogen dependent, of high grade, have various histologies, particularly papillary serous and clear-cell, and have a much poorer prognosis.

#### **1.3.3.1 Pre-cancers of the endometrium**

Endometrial carcinoma develops out of changes found in the endometrial surface epithelium. Both endometrial cancer types (endometrioid and serous cancer) have potentially precancerous precursor lesions preceding such changes. Their detection might be useful in diagnosis and treatment decisions.

Other uterine malignancies which do not necessarily derive or present themselves in the endometrium, such as the group of sarcomas, carcinosarcomas (MMMT) and those of mixed epithelial or mesenchymal origin, have no known regular precursor lesions detectable via endometrial tissue sampling.

However, even for the known endometrial cell alterations at risk of adenocarcinoma - the histologic definition of these precursor lesions and the degree of their risk is subject of ongoing controversy. Currently it is believed that there are – in concordance with the dualistic model of endometrial tumourigenesis also two types of different endometrial precancers which will be described in the following chapters.<sup>15</sup>

**Table 1.2**

	<b>TYPE I</b>	<b>TYPE II</b>
<b>Proportion of endometrial carcinomas</b>	80-90%	10-20%
<b>Age</b>	Pre-/perimenopausal	> 60 yr
<b>Histology</b>	Endometrioid	Papillary serous, clear cell, mixed epithelial
<b>Cell differentiation</b>	Grade I - III	Not applicable
<b>Precursor lesion</b>	Hyperplastic EM	Atrophic EM
<b>Oestrogen stimulation</b>	Dependent	Non-dependent
<b>Clinical behaviour</b>	Indolent	Aggressive

**The dualistic model of endometrial carcinomas - clinical and pathological features.**



## **1.4 Type I endometrial carcinoma**

Synonymous: Endometrioid endometrial carcinoma (EEC).

### **1.4.1 Precancer of type I endometrial carcinoma**

The current classification, adopted by both the WHO and the International Society of Gynecologic Pathologists (ISGP) was proposed by Kurman in 1985.<sup>16</sup> Endometrial hyperplasia can be stratified into 4 groups according to the presence or absence of cytologic atypia and a degree of architectural complexity: 1. simple hyperplasia, 2. complex hyperplasia, 3. simple atypical hyperplasia, and 4. complex atypical hyperplasia. The rationale of this classification is based on the assumption that outcomes are different for the four groups. According to Kurman et al. likelihood of progression to cancer is 1% in patients with simple hyperplasia, 3% of those with complex hyperplasia, 8% of those with simple atypical hyperplasia, and 29% of those with complex atypical hyperplasia.<sup>16</sup> However, reproducibility of the diagnosis of atypical endometrial hyperplasia is known to be less than 40%. Both underestimation and overestimation of the severity of the lesion is very common<sup>17-19</sup> with a prevalence of concurrent carcinoma in women with a biopsy diagnosis of atypical endometrial hyperplasia between 17 and 50%.<sup>20-23</sup> In 2000 the Endometrial Collaborative Group proposed the term, “endometrial intraepithelial neoplasia” (EIN) to characterize early malignant lesions.<sup>24</sup> However, a recent large comparison study of the EIN system versus the WHO system showed similar risks of progression to carcinoma for diagnoses of EIN and atypical hyperplasia.<sup>25</sup>

Developments in our understanding of the molecular genetic changes in pre-invasive endometrial disease call for a more quantitative, less subjective analysis of precancerous

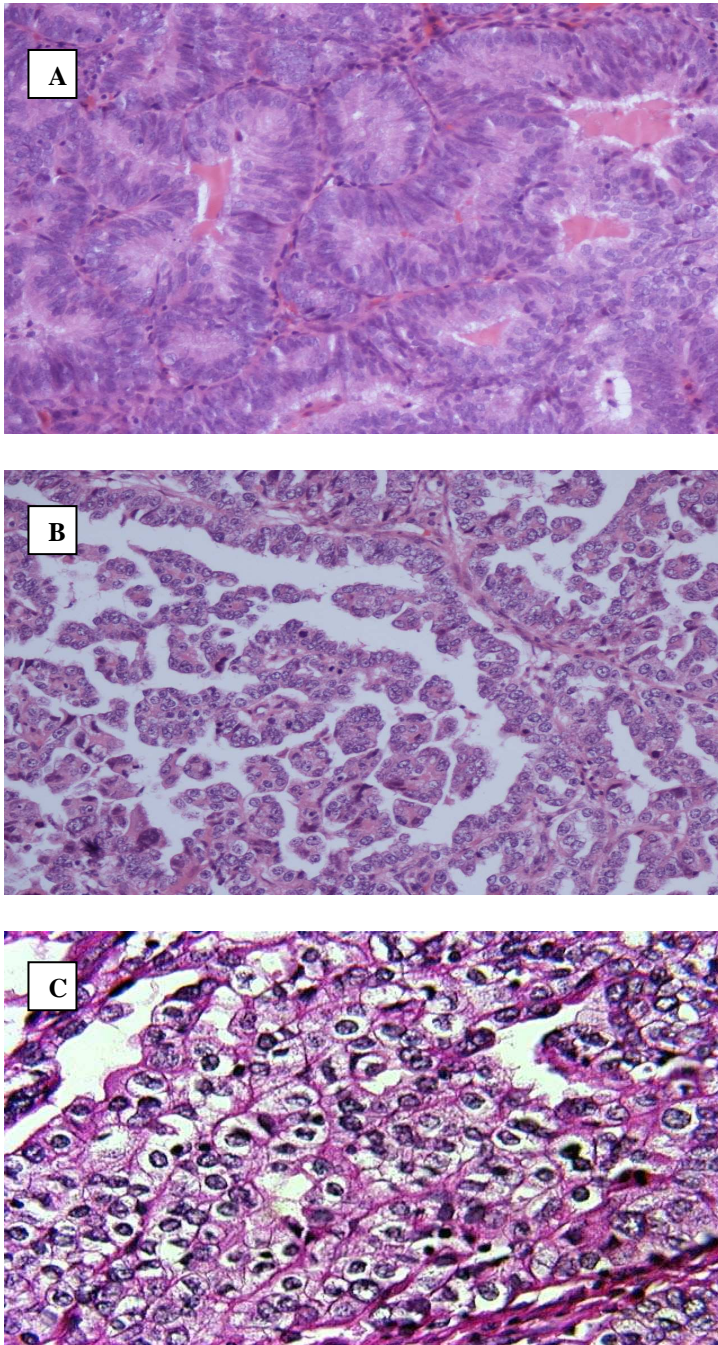
endometrial lesions. The most commonly altered gene is phosphatase and tensin homolog (PTEN) a tumour suppressor gene with an important role in cell survival and possibly in cellular migration and adhesion.<sup>26</sup> PTEN mutations are considered early events in type I tumourigenesis as they are found in up to 20% of endometrial hyperplasia cases (with and without atypia).<sup>27-29</sup> Large scale studies however, have failed to show that loss of PTEN expression has the specificity and reproducibility to be clinically relevant.<sup>30 31</sup> Additional significant and common genetic alterations in type I tumourigenesis include microsatellite instability (MSI) occurring in 20–45% of cases, K-ras mutation (10–30% of cases), and beta-catenin mutation (up to 20% of cases - with or without associated e-cadherin mutations).<sup>32 33</sup> The presence of mutations of the tumour suppressor gene p53 is unusual in endometrioid endometrial carcinoma. However, if present, this may point to high grade histology. It is found in nearly 20% of FIGO grade 3 endometrioid tumours.<sup>34</sup>

None of these markers have so far been able to significantly improve predictability of either endometrial precancerous lesions or an ensuing carcinoma development. In the future however, more readily available immunohistochemical and genetic testing may improve the currently used WHO classification and its predictability.

### **1.4.2 Type I endometrial carcinoma**

Type I endometrial carcinomas represent the majority of sporadic cases of endometrial carcinoma, accounting for 70–80% of new cases which occur predominantly in pre- and perimenopausal women.<sup>35 36</sup> Such cancers are typically of endometrioid type, generally of low-grade, low-stage, and indolent nature (Figure 1.3 A, see page 13). They have a good prognosis with a 5-year survival of more than 95%.

**Figure 1.3:**



**Hematoxylin & Eosin staining, x400, of endometrial carcinoma:**

**A. Endometrioid endometrial carcinoma, grade 1;**

**B. Uterine papillary serous endometrial carcinoma;**

**C. Clear cell endometrial carcinoma.**

Type I endometrial carcinoma commonly express oestrogen and progesterone receptors.<sup>36 37 38</sup> Thus, the associated risk factors are generally pro-oestrogenic, including obesity, high blood pressure, hyperlipidemia, and hyperoestrogenetic situations such as anovulation, nulliparity, infertility, oestrogen secreting tumours, late onset of menopause and endometrial hyperplasia. Long-lasting unopposed oestrogen exposure leads to endometrial hyperplasia, which increases the likelihood of development of atypical hyperplasia and eventually type I endometrial carcinoma. Type I endometrial carcinoma normally resembles proliferative rather than secretory endometrium.<sup>31</sup> Iatrogenic stimulation of the endometrium via tamoxifen- or prolonged oestrogen replacement therapy is another risk factor. Tamoxifen, a selective oestrogen receptor modulator (SERM), is a well known agent in the treatment of oestrogen receptor (ER) positive breast cancer with good evidence to improve both disease-free survival and overall survival.<sup>39</sup> The oestrogen receptor antagonist in the breast shows agonistic effects in the endometrium which is associated with a 2 to 7-fold increased risk of endometrial cancer.<sup>40 41</sup> It is not clear whether there is an association between high or low grade endometrial cancer and tamoxifen use or whether the duration of its use has an influence on endometrial cancer grading. Presently the recommended duration of adjuvant treatment with tamoxifen for patients with breast cancer is 60 months and there are some reports relating the length of tamoxifen use with an increased risk of high risk endometrial cancer.<sup>42 43</sup>

The rare mucinous carcinoma is also considered a type I endometrial carcinoma because they also usually express oestrogen and/or progesterone receptors and are of low histological grade.<sup>12</sup> Immunostaining has been recommended as an adjunct in endometrial carcinoma subtype diagnosis but has not yet found its way into routine

practice. Chapter 1.9.3 will outline molecular characteristics that help to distinguish between type I and II endometrial carcinoma.

## **1.5 Type II endometrial carcinoma**

Synonymous: Non-endometrioid endometrial carcinoma.

### **1.5.1 Precancer of type II endometrial carcinoma**

The sequence of benign epithelium to dysplastic epithelium, to carcinoma in situ, and then to invasive carcinoma is commonly seen in the uterine cervix, breast, prostate, and other organs. In these tumours dysplastic epithelium frequently links the changes between normal epithelium and carcinoma in situ. Whilst the current model for dysplastic, precancerous lesions for type I endometrial cancer appears to be histologically sound and useful, putative precursor lesions to type II cancers are less well understood.

Endometrial intraepithelial carcinoma (EIC) was previously considered as precancerous lesion for uterine papillary serous carcinoma (UPSC) defined as a non-invasive endometrial surface lesion with cytologic features identical to invasive serous carcinoma.<sup>44</sup> However, many reported cases of EIC demonstrate concurrent invasive endometrial serous carcinoma, some of which are associated with intraperitoneal disease without identifiable invasive disease in the corpus. In a recent genome analysis 9 out of 76 uterine serous carcinomas contained concurrent EIC. In all cases a concordant mutation status in *PIK3CA*, *PPP2RIA*, and *TP53* was found.<sup>45</sup> Thus it cannot be considered a precursor which, if diagnosed in time, could yield a therapeutic or even preventative benefit.<sup>46-48</sup> EIC is now considered an early form of UPSC.

Endometrial glandular dysplasia (EmGD) has been suggested to be a potentially better candidate as a precancer to UPSC and clear cell carcinoma (CC).<sup>49</sup> EmGD is characterized by replacing endometrial surface epithelium or its glands with dysplastic cells. It exhibits serous-like differentiation and has cytologic features that are more atypical than “resting endometrium” but cannot be considered a serous endometrial intraepithelial carcinoma (EIC).

EmGD may be found in about 50% of UPSC, but it is rarely found in uteri containing typical endometrioid, i.e. type I endometrial cancer (EEC). It is also mainly found in postmenopausal women. This is in contrast to endometrial hyperplasia and endometrial intraepithelial neoplasia (EIN), which are more commonly associated with and preceding the occurrence of type I endometrial carcinoma lesions.<sup>49 50</sup> Also, EmGD specimen show intermediate degrees of p53 expression which is believed to be one of the most characteristic genetic alterations in type II endometrial carcinoma and UPSC in particular. The presence or absence of p53 may be used to further distinguish EmGD between serous endometrial carcinoma and benign resting endometrium.<sup>51</sup>

However, so far EmGD has not yet reached international recognition as a precancer of any form of type II endometrial cancer which leaves the clinician with virtually no tool to prevent the development of this cancer type and thus is a confounding factor for its poor performance along the diagnosis-treatment-survival axis.

It has been proposed that there is more than only one tumourigenic pathway to reach type II endometrial carcinoma. McConnechy et al. performed mutation profiles from 9 genes described to be associated with the development of type I or II endometrial cancer. They partly found mixed mutation profiles for uterine papillary serous cancers

which suggested different origins and pathways leading to clinico-pathologically similar tumours.<sup>52</sup>

### **1.5.2 Type II endometrial carcinoma**

Type II endometrial carcinomas are far less common. Although these tumours only account for 10-20% of all endometrial malignancies,<sup>53 54</sup> they are responsible for ~50% of all relapses<sup>55-57</sup> and show a low 5-year, all stage, overall survival rate of 35%.<sup>53-58</sup> Histologically they are non-endometrioid, most frequently serous, less frequently clear cell and other histology, always high-grade in differentiation, typically arising in an atrophic endometrial background, and often have deep myometrial penetration. (Figure 1.3 B, see page 13 and Table 1.2, see page 10) They usually occur at an older age, approximately 5 – 10 years later than type I tumours. There is no relationship to oestrogen stimulation. Clinically, type II endometrial carcinomas have an aggressive behaviour, with a high frequency of distant spread to pelvic lymph nodes. They are histologically partly similar to such tumours in the ovary and the fallopian tube, and the prognosis is worse compared to ovarian tumours.<sup>59</sup> Small cell, undifferentiated, squamous cell and carcinomas of mixed histology may also be encountered among type II carcinomas, but little is known about their tumourigenesis.<sup>26</sup>

#### **1.5.2.1 Uterine papillary serous carcinoma (UPSC)**

UPSC is a highly malignant tumour representing about 10% of endometrial carcinoma characterised by a complex papillary serous architecture with tufted stratification of the epithelial lining, high nuclear to cytoplasmic ratio, notable nuclear pleiomorphism, macronuclei, and a high rate of mitosis. Morphologically it appears like an ovarian

serous papillary carcinoma, with similar intra-abdominal spread and frequently coincides with raised serum antigen CA125.<sup>60</sup> (Figure 1.3 B, see page 13) These tumours characteristically do not express oestrogen or progesterone receptors and are often described to overexpress p53.<sup>61</sup>

The overall 5-year survival with a reported 18-27% is very poor. Survival in stage I-II is described to be 35-50%, decreasing to 0-15% in stages III-IV.<sup>56</sup> There has been much debate about whether UPSC has a precursor lesion which might regularly precede the carcinoma thus potentially giving the opportunity for preventative action. Serous endometrial intraepithelial carcinoma (EIC) and endometrial glandular dysplasia (EmGD) have been identified as potential albeit controversial models and are discussed above in detail in chapter 1.5.1. So far there is no accepted precursor lesion which means there is no option to diagnose and treat a certain number of cases before the carcinoma has developed/disseminated and the prognosis is poor. Molecular changes observed in UPSC will be highlighted in chapter 1.9.4.

#### **1.5.2.2 Clear Cell Carcinoma**

About 5% of endometrial carcinoma is of clear cell histology. Clinical behavior and epidemiology is similar to uterine serous cancer. They also occur in the postmenopausal woman, are non-oestrogen dependent and not associated with obesity or diabetes. There is no precursor lesion clearly identified. Like UPSC, clear cell tumours are regarded as aggressive with a propensity for extra-uterine spread or relapse and have been reported to show extra-uterine deposits in the presence of clinically stage I disease occurring even without deep myometrial invasion.<sup>62</sup>



Microscopically, clear cell carcinoma show tubulo-cystic, papillary or solid patterns, can have a clear appearance because of their high glycogen content, and may also include eosinophilic cells and hobnail cells (Figure 1.3 C, see page 13).<sup>63</sup> All cases are graded as poorly differentiated. However, clinical course and survival seem to be slightly better than with UPSC.

Abeler et al. reviewed patients with clear-cell carcinoma and observed a 5-year survival for all stages as 42%, compared with 27% for UPSC.<sup>61</sup> Carcangiu and Chambers reviewed 29 cases of pathological FIGO stage I and II clear-cell carcinoma. They recorded a 5-year survival for stage I disease of 73% and 59% for stage II disease.<sup>56</sup> If surgically staged completely with bilateral pelvic lymphadenectomy survival figures are improving.<sup>64</sup> This obviously is partly due to an upstaging of a proportion of cases. However, Creasman et al. reported a 5-year survival of 81% for surgically staged stage I clear-cell carcinoma compared with 72% for uterine papillary serous carcinoma, and 76% for grade 3 endometrioid cancers.<sup>62</sup> P53 expression levels are reported to be intermediate between those in uterine papillary serous carcinoma and those in endometrioid cancers.<sup>63</sup>

### **1.5.2.3 Tumours of Mixed Histology**

Endometrial carcinomas of mixed histology occur with an incidence of approximately 10%. Given the fact that they are almost as common as UPSC or clear cell carcinoma together they are under-represented in studies and publications for mainly three reasons: They do not fit into the usual group-distinctions, like “endometrioid” or “UPSC”; hence are not classified by clear inclusion criteria. Pathologists may choose to classify them into the histological group dominant in the tumour rather than giving the term “mixed”.<sup>65</sup> There is no uniformity in the literature, what the term “mixed” actually

constitutes – mixed epithelial, mesenchymal etc. Mixed epithelial tumours include various combinations of endometrioid-, clear cell-, and uterine papillary serous carcinoma. Cirisano et al. stated in a study of 574 patients that 18% had different post-operative histological findings compared to pre-operative histology with more than 25% of the tumour volume of ambiguous histology.<sup>60</sup> They also reported that tumours with mixed histology had a clinical behaviour comparable to that of uterine papillary serous carcinoma. However, in their report they did not state cases with mixed histology separately but grouped them under the three histologies; endometrioid, UPSC and CC. No details were given how this differentiation was made.

The proportion of unusual histology needed in a mixed carcinoma to confer a poor prognosis is unclear; some investigators believe that any amount of poor-prognosis histology (uterine papillary serous carcinoma or clear-cell carcinoma) is sufficient, whereas others think that a small focus of poor histology has very little decisive prognostic impact.<sup>66</sup> The 2010 “Dataset for histological reporting of endometrial cancer” of the Royal College of Pathologist recommends to report any proportion of co-existing morphological subtype and stating the approximate percentage even if it comprises less than 10% of the tumour tissue. This is important since the clinical relevance according to the proportion of histological subtype in view of tumour behaviour is so far controversial.<sup>67</sup>

Treatment approaches for tumours of mixed histology, when a known aggressive variant is present, are similar to those for uterine papillary serous carcinoma and clear-cell carcinoma. Adenosquamous uterine carcinoma and adenoacanthoma are also regarded as being of mixed histology. They are believed to be of poor prognosis. However, according to a large study by Pekin et al. they all have a similar prognosis,

suggesting that adenocarcinomas with and without squamous differentiation should be approached in a similar way. The prognosis is dependent on the grade of the glandular component.<sup>68</sup>

### **1.5.3 Summary of Type II endometrial carcinoma**

Although type II carcinomas only account for 10-20% of all endometrial malignancies, they are responsible for ~50% of all relapses and a low 5-year, all stage, overall survival rate of 35%.<sup>58</sup> Clinically, type II endometrial carcinomas have an aggressive behaviour, with a high frequency of distant spread to pelvic lymph nodes. In the absence of an accepted precursor lesion there is no option to diagnose and treat a certain number of cases before the carcinoma has developed.

As it will be outlined below surgical as well as systemic treatment options have so far demonstrated limited impact on the poor prognosis of this group of tumours. Additionally only few markers are available to clearly identify such lesions. A central aim of this study was to identify prognostic markers that can help guide treatment decisions in type 2 tumours.

## **1.6 Management of endometrial carcinoma**

### **1.6.1 Presentation and Diagnosis**

90% of the women who develop endometrial cancer present with bleeding and more than 70% of these are postmenopausal.<sup>15</sup> 5%-15% of postmenopausal women with abnormal bleeding will have endometrial carcinoma. This obvious symptom encourages many women relatively soon after onset of these symptoms to seek medical help.<sup>69</sup> Thus approximately 75% of all patients with endometrial cancer present with early stage disease confined to the body of the uterus. Other symptoms can include low pelvic pain, vaginal discharge or, in advanced cases, urinary or rectal symptoms. Not uncommonly, diagnosis is made following investigations after endometrial dysplastic cells were found in a cervical smear. Distant metastatic disease is unusual at diagnosis although local metastases to the lower vagina can lead to a patient presenting with vulvo-vaginal soreness or bleeding. In the United Kingdom and most other developed countries patients will be referred to a gynaecological department or a gynaecological cancer unit where they will undergo detailed investigation.

The diagnostic workup usually includes clinical examination, ultrasound imaging and hysteroscopy/endometrial biopsy. Whilst transvaginal ultrasound has a sensitivity of >90% to detect endometrial abnormality it is non-specific.<sup>69 70</sup> In the UK, women presenting with postmenopausal bleeding plus showing an endometrial thickness of >5 are recommended to undergo further investigations such as hysteroscopy/endometrial biopsy. However, more recent guidelines such as the one from the Scottish Intercollegiate Guidelines Network (SIGN) recommend a cut-off threshold of 3mm.<sup>71</sup>

The office based Pipelle biopsy to obtain tissue for histology is also a reliable tool with a reported sensitivity to detect endometrial cancer of over 95 %.<sup>72 73</sup>

### **1.6.2 Staging**

Endometrial cancer is graded and staged on the hysterectomy specimen according to FIGO (International Federation of Gynaecology and Obstetrics) rules. Although the FIGO staging has been updated in 2009, the older staging described below will obviously still be found in the literature.<sup>2</sup>

The main difference is that the old FIGO staging system divided stage I into three rather than two sub-stages: Stage IA, tumour limited to endometrium; IB, tumour invades up to less than half of myometrium; IC, tumour invades to more than one half of myometrium.<sup>74</sup> In this study patients were staged within the old system since diagnosis and treatment predated the introduction of the new system. Table 1.3 (see page 24) outlines the new staging system in detail.<sup>74</sup>

**Table 1.3**

<b>FIGO STAGES</b>		<b>TNM CATEGORIES</b>
	Primary tumour cannot be assessed	TX
	No evidence of primary tumour	T0
<b>0</b>	Carcinoma in situ (pre-invasive carcinoma)	Tis
<b>I*</b>	Tumour confined to the corpus uteri	T1
<b>IA*</b>	No or less than half of the endometrium	T1a
<b>IB*</b>	Invasion equal to or more than half of the myometrium	T1b
<b>II*</b>	Tumour invades cervix but does not extend beyond uterus**	T2
<b>III*</b>	Local and/or regional spread as specified in IIIA, B, C	T3 and/or N1
<b>IIIA*</b>	Tumour involves serosa and/or adnexae (direct extension or metastasis) and/or cancer cells in ascites or peritoneal washings) #	T3a
<b>IIIB*</b>	Vaginal involvement (direct extension or metastasis)#	T3b
<b>IIIC*</b>	Metastasis to pelvic and/or para-aortic lymph nodes#	N1
<b>IIIC1*</b>	Positive pelvic nodes	
<b>IIIC2*</b>	Positive para-aortic lymph nodes with or without positive pelvic lymph nodes	
<b>IV*</b>	Tumour invades bladder and/or bowel mucosa, and/or distant metastases	
<b>IVA*</b>	Tumour invades bladder- and/or bowel mucosa	T4
<b>IVB*</b>	Distant metastasis (excluding metastasis to vagina, pelvic serosa, or adnexa, including metastasis to intra-abdominal lymph nodes other than para-aortic and/or inguinal nodes)	M1

**2009 FIGO staging system for endometrial carcinoma. \*, either G1, G2, or G3; \*\*, endocervical glandular involvement only should be considered as stage I and no longer as stage II; #, positive cytology has to be reported separately without changing the stage.**

### 1.6.3 Grading

The histopathological degree of tumour differentiation (grading) has an important impact on the natural history of this disease and on treatment selection. Before the grade is determined all tumours are to be microscopically verified and classified as outlined in Table 1.1 on page 8. Endometrioid carcinomas are then graded into:

- **Gx – Grade cannot be assessed;**
- **G1 – Well differentiated;**
- **G2 – Moderately differentiated;**
- **G3 – Poorly or undifferentiated.**

According to the system of the International Federation of Gynecology and Obstetrics (FIGO), an endometrioid carcinoma of grade 1 consists of well-formed glands, with no more than 5% solid non-squamous areas. Squamous differentiation is not regarded as solid tumour growth. Grade 2 carcinomas consist of 6–50% and those of grade 3 of more than 50% solid non-squamous areas.<sup>75</sup> Conspicuous cytological and nuclear atypia will upgrade a tumour from grade 1 to 2, or from grade 2 to 3.

It is important to note that according to FIGO, only endometrioid carcinomas are grouped with regard to the degree of differentiation. All other cases of carcinoma of endometrial cancers such as the typical type II cancers, UPSC, CC and tumours of mixed histology, are regarded as grade 3 (G3). Sarcoma and carcinosarcoma are staged and graded separately.

## **1.6.4 Treatment of endometrial carcinoma (type I and type II)**

### **1.6.4.1 Type I endometrial carcinoma**

#### **1.6.4.1.1 Surgery**

Early stage endometrioid endometrial carcinomas (type I) which reveal localised disease are usually curable and treated with the removal of the uterus and ovaries with peritoneal washings. This achieves excellent local control of disease. The International Federation of Gynecology and Obstetrics (FIGO) mandates a pelvic and para-aortic lymphadenectomy for endometrial cancer staging, however, two recently published large randomized controlled trials found no survival benefit in routine pelvic lymphadenectomy in endometrioid endometrial cancer.<sup>76 77</sup> Internationally and even within the UK there is considerable variation in the practice and extent of lymphadenectomy in endometrial cancer.

#### **1.6.4.1.2 Adjuvant therapy in type I endometrial carcinoma**

Most women with early-stage EEC recur in the vagina or pelvis. In type I endometrial cancer, adjuvant therapy is increasingly focused locally or even deferred depending on risk factors. The recently published PORTEC II trial comparing vaginal brachytherapy (VBT) versus pelvic external beam radiotherapy (EBRT) in prevention of vaginal recurrence in an intermediate risk cohort of 427 patients did not show any difference in disease free survival or overall survival but proved that VBT is equally effective as EBRT with fewer gastrointestinal toxic effects.<sup>78</sup>

In type I endometrial carcinoma cytotoxic chemotherapy has a limited place in the management of advanced or recurrent endometrial cancer. Whilst no one drug or regimen offers a clear benefit for women with advanced endometrial cancer, platinum drugs, anthracyclines and paclitaxel seem the most promising agents.<sup>79</sup>



#### 1.6.4.1.3 Hormone therapy

In type I endometrial cancers with hormone receptor positivity hormone therapy is an option although patients are rarely curable and there is no standard therapy. It has also been used as an alternative treatment for a small subset of patients mainly with simultaneous regional and distant disease particularly if comorbidity excludes them from surgical intervention. Progestin therapy is also given when endometrial cancer recurs, although response rates are low.

### **1.6.4.2 Type II endometrial carcinoma**

#### 1.6.4.2.1 Surgery

In type II endometrial cancer (UPSC, CC and tumours of mixed histology) the initial management for the majority of women is surgical exploration and comprehensive staging, including total abdominal hysterectomy and bilateral salpingo-oophorectomy, pelvic washings, lymphadenectomy and omentectomy. In addition to providing prognostic information, accurate identification or exclusion of metastatic disease is important for the individual planning of adjuvant therapy and surveillance. In many studies lymphadenectomy is believed to provide a therapeutic benefit in women with high-grade endometrioid endometrial cancer, and particular type II disease.<sup>65</sup> Unfortunately, the above mentioned ASTEC-trial as well as the trial by Panici at al. were underpowered to be able to make a valid statement about the value of lymphadenectomy in high-risk patients. In above trials only 4% and <1% (respectively) of the study population had UPSC histology and no individual analysis for this part of the population was performed.

#### 1.6.4.2.2 Neoadjuvant chemotherapy

Neoadjuvant chemotherapy has been described as a successful option in select cases who might be poor candidates for upfront surgery or where pre-treatment imaging might be suggestive of disseminated disease.<sup>80 81</sup>

#### 1.6.4.2.3 Risk and pattern of recurrence

Surgical staging studies have defined the spread pattern of EEC, i.e. type I endometrial carcinoma.<sup>82</sup> Pathologic findings associated with increased risk of nodal metastasis, as well as disease recurrence, include tumour grade, depth of myometrial invasion, positive peritoneal cytology, tumour within the isthmus-cervix, adnexal involvement, and LVSI.<sup>83</sup> Women with type I endometrial carcinoma are commonly stratified based upon these features into groups at low, intermediate, and high-risk for recurrence of disease. Unfortunately, the typical features of type I endometrial cancer risk prediction such as myometrial invasion or lymphovascular space invasion, are not reliable in type II cancers to assess the risk of metastatic disease. Numerous investigators utilizing comprehensive staging have documented that the majority of women with type II endometrial cancer have a high risk of relapse and metastatic disease even in the absence of such “high-risk” pathologic features.<sup>48 83-88</sup> Hui et al. found extrauterine disease in 38% of comprehensively staged women whose uterine disease was confined to an endometrial polyp.<sup>89</sup> Slomovitz et al. needed to upstage 30 in 129 cases of UPSC from stage I to III solely due to positive lymphadenectomy findings.<sup>84</sup>

The majority of UPSC patients relapse outside of the pelvis, often in multiple sites. Since type I and type II endometrial carcinoma show such different patterns and frequency of disease, there is a need for different adjuvant management plans.

#### 1.6.4.2.4 Adjuvant Therapy in type II endometrial carcinoma

Type II endometrial cancer, in particular UPSC and CC have the tendency to recur within the peritoneal cavity, hence most investigations into the role of radiotherapy for early-stage adjuvant treatment for these cancers focus on whole abdominal radiotherapy with or without a pelvic boost (WAPI).<sup>90-93</sup> Above mentioned PORTEC II trial was underpowered to make any statement about the value of VBT or EBRT in high risk cases such as G3 EEC. UPSC/CC were not included. There have been studies comparing adjuvant radiotherapy with or without combination with chemotherapy but none have produced encouraging findings to support a major role for radiotherapy in the treatment of type II endometrial cancer.<sup>93</sup>

Optimum treatment for type II endometrial is unclear, particularly in early disease. Unfortunately, there have been no randomized trials exploring the potential utility of adjuvant chemotherapy for women with stage I UPSC/CC. While five-year survival in a single institution series of 27 women with surgical stage I UPSC was reported to be 62.9% overall, the prognosis of women confirmed to have stage IA disease appeared to be relatively favourable.<sup>84</sup> There were even a number of studies advocating observation only in stage IA disease.<sup>55 94 95</sup> Other groups, however, reported significant and rapid recurrence even in stage IA UPSC disease whether chemotherapy was given or not.<sup>96</sup>

The high frequency of distant recurrence in early stage UPSC, along with treatment failures within the radiation fields, has led to increasing use of adjuvant chemotherapy and reports of its success. Fader et al. investigated retrospectively 141 patients and showed that recurrence and survival outcomes were significantly improved in patients who received platinum/taxane chemotherapy  $\pm$  radiotherapy compared to women who received no adjuvant therapy or radiotherapy alone.<sup>96</sup> Women treated with platinum/taxane-based chemotherapy had a significantly lower recurrence rate (11.2%)

when compared to patients who did not receive chemotherapy (26.9%;  $p = 0.021$ ). This effect was most pronounced in women with stage IB/IC UPSC. Also, the use of intraperitoneal chemotherapy has been reported for UPSC. Chambers et al. treated 13 women with UPSC of stage IA–IVB with intraperitoneal cisplatin along with intravenous doxorubicin and cyclophosphamide. The 3-year overall survival of 24.1% was similar to women treated at the same institution with intravenous cyclophosphamide, doxorubicin, and cisplatin.<sup>97</sup>

Patients with type II endometrial cancer frequently relapse. Treatment options at advanced or recurrent disease are limited and consist of palliative chemotherapy with platinum agents or taxanes.

The Gynecologic Oncology Group (GOG) conducted a series of phase III randomized prospective trials of chemotherapy for advanced-stage or recurrent endometrial carcinoma.<sup>98-101</sup> The current “gold standard” treatment of advanced-stage or recurrent endometrial carcinoma is based upon the results of these trials. In the most recently completed GOG studies, the addition of paclitaxel to cisplatin and doxorubicin (TAP) following surgery (cytoreduction to less than 2 cm maximal residual disease) and radiation (tumour volume directed) was not associated with significant improvement in recurrence-free survival but was associated with greater toxicity in women with advanced stage endometrial cancer.<sup>102</sup> The GOG concluded that despite the apparent biological differences between endometrioid and serous endometrial cancers, the overall response rates (40-50%) to taxane, doxorubicin and platinum chemotherapy and survival after such treatment is similarly poor for women with advanced or recurrent UPSC as for those with advanced or recurrent endometrial cancer of other histologies.

#### 1.6.4.2.5 Hormone Therapy

In cases of type II endometrial carcinoma progesterone treatment has no effective role.

#### 1.6.4.2.6 Summary of treatment of type II endometrial cancer

Although type II endometrial cancer comprises of 10-15% of all endometrial carcinoma cases, it accounts for a disproportionately high number of endometrial cancer related deaths. Whilst type I endometrial carcinoma is usually diagnosed at an early stage, is often curable and long term survival favourable, type II cancers often present already with extra-uterine disease which sometimes can only be identified with comprehensive surgical staging. Despite improvements in surgery such as cytoreduction and lymphadenectomy the incidence of both local and distant recurrence is high among women with stage I UPSC/CC compared to most women with EEC.

In type II endometrial cancer adjuvant platinum/taxane-based chemotherapy with or without radiotherapy appears to improve progression-free and overall survival outcomes only in the comparatively rare early-stage patients. Traditional therapies are not associated with long-term survival in women with more advanced or recurrent disease. Whatever the receptor status might be, hormone therapy seems to have no role in type II endometrial carcinoma.

Altogether, treatment options in type II endometrial cancers are largely limited to aggressive surgical- and/or chemotherapy regimen, which have not altered the poor prognosis of these tumours. There is an unmet clinical need for robust prognostic markers that can help guiding therapeutic decisions in these endometrial cancer tumour types, particularly at recurrence and in advanced stage tumours and to identify new therapeutic targets.

## 1.7 Uterine sarcoma

Uterine sarcomas arise from mesenchymal tissue (e.g. connective tissue, smooth muscle) and account for 3 - 7% of uterine malignancies. They are rare, occur at a mean age of 60, behave generally more aggressively and show a poorer prognosis than endometrioid endometrial cancers with a 5-year survival rate of ca 40%.<sup>103</sup> Table 1.4 (see below) lists the most common types of histology in uterine sarcomas.

**Table 1.4**

	<b>CLASSIFICATION OF SARCOMA</b>	
<b>1.</b>	<b>Carcinosarcoma or MMMT</b>	<b>(40-50%)</b>
<b>2.</b>	<b>Leiomyosarcoma</b>	<b>(30-40%)</b>
<b>3.</b>	<b>Endometrial stromal sarcoma (ESS)</b>	<b>(10-15%)</b>
<b>4.</b>	<b>Adenosarcoma</b>	<b>(&lt;10%)</b>
<b>5.</b>	<b>Undifferentiated endometrial sarcoma</b>	<b>(5%)</b>
<b>6.</b>	<b>Smooth-muscle tumours of uncertain malignant potential (STUMP)</b>	<b>rare</b>

**WHO classification of the most common types of uterine sarcoma, (%) = percentage among sarcoma group.**

“Typical” sarcoma types are the leiomyosarcoma, endometrial stromal sarcoma (ESS) and undifferentiated endometrial sarcoma and are described together. There were no cases of STUMP and adenosarcoma in the cohort of this study, thus they are not described further. For reasons indicated in chapter 1.2 carcinosarcoma is described in chapter 1.8.

### **1.7.1 Presentation and diagnosis of sarcomas**

Some sarcomas present with vaginal bleeding with or without odorous discharge and may be accompanied with abdominal distension or pelvic pressure symptoms (urinary frequency, constipation etc.) and pain. Leiomyoma and leiomyosarcoma are difficult to distinguish but if they are symptomatic in a postmenopausal state or with disproportional vaginal bleeding the suspicion should be raised. Only exceedingly rarely does a leiomyoma degenerate into a sarcoma. The diagnosis of uterine sarcoma is based on histological examination of the believed myomatous tissue or the uterine specimen. Uterine curettings alone are not reliable.

### **1.7.2 Staging of sarcomas**

Table 1.5 (see page 34) shows the FIGO staging system for uterine sarcoma (2009).<sup>1</sup>

**Table 1.5**

STAGE	DEFINITION
	<b>(1.) Leiomyosarcoma and Endometrial Stromal Sarcoma (ESS)</b>
<b>I</b>	<b>Tumour limited to uterus</b>
IA	Less than or equal to 5 cm
IB	More than 5 cm
<b>II</b>	<b>Tumour extends beyond the uterus, within the pelvis</b>
IIA	Adnexal involvement
IIB	Involvement of other pelvic tissues
<b>III</b>	<b>Tumor invades abdominal tissues (not just protruding into the abdomen)</b>
IIIA	One site
IIIB	More than one site
IIIC	Metastasis to pelvic and/or para-aortic lymph nodes
<b>IV</b>	
IVA	Tumour invades bladder and/or rectum
IVB	Distant metastases
	<b>(2) Adenosarcomas</b>
<b>I</b>	<b>Tumour limited to uterus</b>
IA	Tumour limited to endometrium/endocervix with no myometrial invasion
IB	Less than or equal to half myometrial invasion
IC	More than half myometrial invasion
<b>II</b>	<b>Tumour extends beyond the uterus, within the pelvis</b>
IIA	Adnexal involvement
IIB	Tumour extends to extrauterine pelvic tissue
<b>III</b>	<b>Tumour invades abdominal tissues (not just protruding into the abdomen).</b>
IIIA	One site
IIIB	More than one site
IIIC	Metastasis to pelvic and/or para-aortic lymph nodes
<b>IV</b>	
IVA	Tumour invades bladder and/or rectum
IVB	Distant metastasis
	<b>(3) Carcinosarcomas</b>
	Carcinosarcomas should be staged as carcinomas of the endometrium.

**FIGO staging system for uterine sarcoma (2009) <sup>1</sup>**



### **1.7.3 Management of sarcomas**

Total abdominal or laparoscopic hysterectomy (TAH/TLH) and bilateral salpingo-oophorectomy (BSO) is the standard surgical treatment and staging. Radical surgery is of no benefit. Peritoneal washings and biopsies from areas of suspected metastases are part of the staging. Cytoreduction in case of tumour spread outside the pelvis has not been proven beneficial. Routine pelvic and or para-aortic lymphadenectomy is not required in leiomyosarcoma and undifferentiated sarcoma. This has been suggested for ESS but it is still under investigation. Obviously, visibly enlarged nodes may be biopsied. Preservation of the ovaries only in young women may be safe in early-stage leiomyosarcoma whilst it is uncertain in ESS and undifferentiated sarcomas.

As to adjuvant therapy, radiotherapy (RT) has demonstrated a reduction in local recurrence (LR) and improvement in progression-free survival (PFS) in uterine sarcoma but the overall survival (OS) benefit is unclear. Adjuvant chemotherapy is commonly recommended for women with resected stage I to IVa disease, particularly those at high-risk for recurrence.

#### **1.7.3.1 Endometrial stromal sarcoma (ESS)**

Hormonal therapy is the mainstay of adjuvant therapy and associated with improved overall survival for oestrogen and progesterone receptor-positive ESS although agents and regimen vary. Hormone therapy may also be used in other ER/PR-positive uterine sarcomas. Commonly, GnRH-analogues are prescribed for pre-menopausal women and aromatase inhibitors (exemestane, anastrozole, letrozole) or progestins (e.g. medroxyprogesterone acetate (MPA) or megestrol acetate) for post-menopausal women. There is some suspicion that tamoxifen might be associated with tumour recurrence. Aromatase inhibitors may have therapeutic value. Adjuvant radiotherapy may not be

indicated for a tumour with such good prognosis in early disease but may be used in advanced stage to control local disease.

#### **1.7.3.2 Leiomyosarcoma**

Regarding adjuvant chemotherapy, the combination of doxorubicin or docetaxel / gemcitabine is currently a recommended regimen which showed some survival benefit in a small prospective trial.<sup>104</sup> The benefit of radiotherapy (RT) has not been reliably proven, possibly due to its tendency for distant metastases. It may be used in situations of local recurrence or the assumed risk for it. Some patients may respond to hormonal therapy.

#### **1.7.3.3 Undifferentiated sarcoma**

It is suggested to apply adjuvant RT for patients with a high risk of local recurrence.

There is no consensus about adjuvant chemotherapy for undifferentiated endometrial sarcoma. A platinum based combination chemotherapy might be a reasonable option.

### **1.7.4 Prognosis of sarcomas**

Uterine sarcomas as a whole carry a significantly poorer prognosis as compared to other gynaecologic malignancies. In stage I uterine sarcomas have a five-year survival rate of 76 %, falling to 60, 45, and 29 % for stages II, III and IV. Tumour stage, degree of resection, grading, and mitotic count are believed to correlate with prognosis. Postmenopausal women are said to do worse than pre-menopausal women. ESS as an exception is believed to have a fairly good prognosis whilst leiomyosarcomas have a worse prognosis relative to other uterine sarcomas.

### **1.7.5 Summary of uterine sarcoma**

Sarcomas are as a group of uterine malignancies, with the exception of the STUMP, of particularly poor prognosis. Their histologic and prognostic diversity as well as relative rarity, render large trials to optimize diagnosis, prognosis or treatment difficult to conduct. New molecular markers may open new ways to individualise prognosis and possibly improve treatment.

## **1.8 Carcinosarcoma**

Malignant mixed mullerian tumour = MMMT. Uterine carcinosarcoma are a biphasic neoplasm distinctly and separately composed of both carcinomatous (epithelial) and sarcomatous (connective tissue, i.e. stromal) elements. It comprises of only 1% of uterine neoplasms but accounts for 40% of all sarcomas.<sup>105</sup>

### **1.8.1 Presentation and diagnosis of carcinosarcomas**

Carcinosarcoma is a rare and aggressive tumour with 30% of women showing extra-uterine disease by the time of diagnosis. It is diagnosed at a median age of 65 years. The serum level of CA125 is usually elevated. It carries a similar risk profile when compared to endometrial cancer: obesity, nulliparity, exposure to oestrogen and history of tamoxifen use.<sup>106</sup>

Patients often present with vaginal bleeding and a polypoid mass filling the endometrial cavity. Both histological components may display different histologies. The epithelial component may be an endometrioid, serous, clear cell adenocarcinoma; squamous or undifferentiated. The stromal component may be of uterine origin (endometrial, leiomyosarcoma or undifferentiated) - these tumours are termed homologous. If the stromal component is of any other connective tissue (chondrosarcoma, osteosarcoma, etc.) it is termed a heterologous tumour. The most common form consists of a high-grade serous papillary carcinoma and an endometrial stromal sarcoma. In metastatic sites the epithelial component seems to play the dominant role, hence metastatic carcinosarcoma often display a clinical behaviour similar to uterine papillary serous cell

carcinoma or epithelial ovarian carcinoma.<sup>107</sup> The diagnosis is made at histology and often only after hysterectomy. Approximately 40% of endometrial biopsies will not reveal the correct diagnosis.

### **1.8.2 Staging of carcinosarcoma**

Carcinosarcoma is staged according to the FIGO staging system for endometrial carcinoma. See Table 1.3 on page 24.

### **1.8.3 Management of carcinosarcoma**

TAH and BSO with peritoneal washings is the standard of care. Some clinicians will advocate an omentectomy due to the risk of upper abdominal disease. The incidence of regional lymph node metastases is approximately 20%. Pelvic and para-aortic lymphadenectomy will complete the staging procedure and seems to carry a significant survival benefit.<sup>107</sup>

The recurrence rate is approximately 60%. The role of adjuvant chemo- and radiotherapy is uncertain. Whilst brachytherapy or external beam radiotherapy seems to benefit local control it may not improve disease-free survival or overall survival. Adjuvant chemotherapy has yet to prove its effectiveness. The approach is like that for high grade endometrial carcinomas. Cisplatin, ifosfamide, doxorubicine and taxanes are often mentioned choices of agents with various combinations.<sup>108 109</sup> In view of the poor prognosis even with early stage disease, sequential or multimodality therapy regimens have been introduced including chemotherapy and pelvic as well as whole body

radiotherapy. Favourable results have been reported anecdotally. However, there are no randomized trials available.

#### **1.8.4 Prognosis and summary of carcinosarcoma**

Carcinosarcoma are the smallest group of uterine malignancies and like sarcoma of particularly poor prognosis. The overall 5-year survival for patients with carcinosarcoma is reported to be 25 - 35% and for those with stage I (confined to the corpus) ca 50%.<sup>105 109</sup> This is in contrast with that of endometrial cancers for which 5-year survival in stage I disease is approximately 80% or better.<sup>110-112</sup> Recurrences tend to occur within the first year following treatment. Their general rarity as well as their vast diversity of biphasic histological composition, render clinical trials difficult to conduct but studies which include carcinosarcoma are necessary on the quest to find potential prognostic and/or therapeutic markers to improve outcome. New molecular markers may open new ways to individualise prognosis and possibly improve treatment.

## **1.9 Translational research and endometrial carcinoma**

In this chapter translational research will be defined, molecular markers which are characteristic of type I and II endometrial cancer will be outlined and novel markers currently investigated in trials highlighted in chapter 1.10. Chapter 1.11 will then introduce the novel markers that were investigated in this study.

### **1.9.1 Definition**

The Translational Research Working Group of the American National Cancer Institute defines: "Translational research transforms scientific discoveries arising from laboratory, clinical, or population studies into clinical applications to reduce cancer incidence, morbidity, and mortality."<sup>113</sup> One role of translational medicine is to provide biomarkers which enhance decision making at critical milestones in the development or treatment process of a disease. A biomarker is a quantifiable biological variable that characterises cellular, organ, physiological, pathological or clinical condition. For example a biomarker could be (amongst others) a gene or protein expression or biochemical profile or a quantifiable measurement from an image.

### **1.9.2 Rationale for molecular medicine and novel therapies in endometrial cancer**

Current understanding is that the 'umbrella-diagnosis' of endometrial cancer encompasses a heterogeneous disease with grossly different biology and therefore

oncologic behaviour, which demands more individualised treatment. Endometrial carcinoma develops as the result of an accumulation of alterations in cellular regulatory pathways, such as oncogene activation and tumour suppressor gene inactivation which leads to dysfunctional cell growth. Some of these molecular alterations appear to be more specific in type I, some in type II endometrial cancers.<sup>114</sup>

Advanced understanding of the molecular mechanisms in cancer development, progression and metastasis has unveiled a spectrum of targets for a more precise understanding and definition of the diseases in question (type I and II endometrial cancer), i.e. a “molecular profile” and the subsequent therapeutic intervention.<sup>115</sup>

Table 1.5 (see page 34) will outline a summary of markers which are currently used to distinguish between type I and type II endometrial cancers. It is recommended to use a panel of markers for that purpose for none of these markers are sufficiently sensitive or specific enough to function alone in a binary way. Chapter 1.9.3 will outline these markers in more detail.



**Table 1.6**

<b>MARKER</b>	<b>TYPE I ENDOMETRIOID CARCINOMA</b>	<b>TYPE II NON-ENDOMETRIOID CARCINOMA</b>
<b>ER/PR</b> expression	<b>Frequent</b> (60-95%) <sup>116 117-119</sup>	Less frequent? (9-54%) <sup>120</sup>
<b>pTEN</b> mutation Inactivation (loss)	<b>Frequent</b> (-90%) <sup>121 122</sup>	Rare/Less frequent (-10%) <sup>38 123</sup>
<b>P16</b> mutation Inactivation	Less frequent (10%) <sup>38</sup>	<b>Frequent</b> (40%) <sup>38</sup>
<b>P53</b> mutation overexpression	Less frequent (10-20%) <sup>38</sup>	<b>Frequent</b> (70-90%) <sup>38 45 123 124</sup>
<b>K-ras</b> mutation	<b>Partially</b> (10-30%) <sup>32 35</sup>	Rare/less frequent (0-8%) <sup>35 38 125 126 52</sup>
<b>Erb B2 amplification</b> (Her2-neu overexpression)	Rare (2-5%) <sup>38</sup>	<b>Frequent</b> (26%) <sup>38</sup>
<b>PIK3CA</b> mutation overexpression	<b>Frequent</b> (30%) <sup>127</sup>	(Less) frequent? (5-27%) <sup>45 52 127</sup>
<b>Microsatellite Instability</b> (MSI)	<b>Frequent</b> (10-40%) <sup>38 128</sup>	Rare (0-5%) <sup>38 129 130</sup>
<b>β-Catenin</b> (CTNNB1) overexpression	<b>Frequent</b> (25-40%) <sup>38</sup>	Rare (0-5%) <sup>38 53</sup>
<b>E-Cadherin</b> overexpression	Less frequent (10-20%) <sup>38</sup>	<b>Frequent</b> (80-90%) <sup>38</sup>
<b>PPP 2R1A</b> mutation Inactivation (loss)	Less frequent (7%) <sup>52</sup>	<b>Frequent</b> (ca 40%) <sup>52</sup>
<b>ARID 1A</b> mutation (loss of protein BAF250a)	<b>Frequent</b> (20-47%) <sup>52 131</sup>	Less frequent? (0-11%) <sup>52 131</sup>

**Molecular features of endometrial carcinoma type I and II. ER/PR = oestrogen receptor/progesterone receptor; pTEN = Phosphatase and tensin homolog; p16 = Cyclin-dependent kinase inhibitor 2A; P53 = Tumour protein 53; K-ras = GTPase encoded by the KRAS gene; Erb B2 = HER2 (Human Epidermal Growth Factor Receptor 2); PIK3CA = Phosphatidylinositol 3'-kinase catalytic subunit; PPP2R1A= subunit of protein phosphatase 2; ARID 1A = AT-rich interactive domain-containing protein 1A.**

### **1.9.3 Molecular characteristics of type I endometrial carcinoma**

#### **1.9.3.1 Roles of oestrogen and progestins**

Type I endometrial carcinoma are oestrogen receptor (ER) and progesterone receptor (PR) positive in 60-95% of cases.<sup>116 117-119</sup>

Oestrogen and progestins have largely reciprocally opposing roles on hormonally responsive endometrial tissue. Progestins exert a direct effect on co-existing oestrogenic action by down-regulating the oestrogen receptor (ER). For this reason, the combined biologic effects of circulating progestins and oestrogens are dominated by the progestational component. Large-scale profiling of endometrial tissue RNAs have demonstrated that type 1 endometrioid carcinoma mirrors the protein expression profile seen in oestrogen-driven proliferative endometrium and lacks expression of those genes induced by progestins. A number of genes are not or underexpressed amongst which are tumour-suppressor genes affected by primary mutation or deletion. Downstream target genes are also affected. Another concept is oestrogenic promotion of cell proliferation and inhibition of apoptosis with concomitant modulation of tumour suppressor function. Whilst endometrial cell-proliferation is promoted so is PTEN expression to regulate mitosis and apoptosis. However, in the absence of PTEN-opposing progestins not only normal but also mutant forms of PTEN are greatly increased which consecutively stop counterbalancing proliferation thus promote tumour development.<sup>132 133</sup>

Oestrogen also has a very interesting role in mediating increased cancer risk via down-regulating 'cyclins', a cyclin-dependent kinase binding protein. In normal endometrial

tissue cables is upregulated and kept in balance by progesterone, effectively slowing down cell proliferation. In the majority of endometrial cancers however, cables is absent, possibly downregulated during tumourigenesis by unopposed oestrogen. In support of this theory it has been demonstrated that mutant mice deficient in cables develop hyperplasia and cancer in early age.<sup>134</sup> Whilst it is known that progesterone plays a key role in counter-balancing the proliferative role of oestrogen and its absence results in endometrial hyperplasia and endometrial cancer, its precise actions are complex and not fully understood. Like oestrogen, progesterone acts on its specific receptor. Progesteron receptors (PR) with its isoforms PR-A and PR-B are present in both endometrial stroma and epithelium but their expression status fluctuates according to the hormonal status and other factors.

In type I endometrial cancers ER and PR status are characteristically positive which is indicative of a less aggressive tumour and predictive of a favourable response to progestin therapy.

#### **1.9.3.2    Microsatellite instability**

Microsatellites are short segments of repetitive DNA bases found throughout the genome and mostly in non-coding DNA. During replication DNA repair errors take place leading to small alterations in these short segments or microsatellites. The progressive accumulation of these alterations at microsatellite loci is called microsatellite instability (MSI). If MSI occurs in important regulatory genes it may promote carcinogenesis and is known to play an important role in sporadic colon cancers and in several non-colonic tumours. Patients with known MSI, also called Lynch-Syndrome are believed to have in addition to colon-cancer risk a lifetime risk for

endometrial cancer between 40 and 60% corresponding to a relative risk of 13–20%.<sup>135</sup> MSI is found in 17–25% of sporadic type I endometrial carcinoma<sup>128</sup>, but is rarely present in type II tumours.<sup>129 130</sup>

### **1.9.3.3 PTEN – tumour suppressor gene**

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a protein which in humans is encoded by the PTEN gene.<sup>136</sup> PTEN acts as a tumour suppressor gene through the action of its phosphatase protein product. It removes phosphate groups from key phosphoinositide signalling molecules, thus inhibiting AKT and its downstream signalling product mTOR.<sup>137</sup> Decreased activity or loss-of-function mutations of PTEN lead to the activation of multiple signalling pathways, including the PI3K/Akt/mTOR pathway, which affects cell proliferation, apoptosis and migration.<sup>121 122</sup>

Mutations of PTEN have been found in several other tumours such as prostate cancer and glioblastoma. Up to 80% of cases of endometrioid carcinoma reveal a loss of PTEN expression, mainly due to mutations, and to a lesser extent to a loss of heterozygosity (LOH).<sup>121 122</sup> Mutations of PTEN have also been described in endometrial hyperplasia with and without atypia.<sup>27 29</sup> Given the role of endometrial hyperplasia as the putative precursor of type I tumours, PTEN mutations are presumed to play an early role, although probably not a determining step, in tumourigenesis.

### **1.9.3.4 $\beta$ -Catenin (CTNNB1)**

The CTNNB1 gene is located on chromosome 3p21 and encodes the 88 kDa protein  $\beta$ -catenin. Alpha ( $\alpha$ ), beta ( $\beta$ ), and gamma ( $\gamma$ ) catenins belong to a family of structurally

related cytoplasmatic proteins according to their electrophoretic mobility.  $\beta$ -catenin appears to be important in the functional activities of both APC (adenomatous polyposis coli) and E-cadherin. It is a component of the E-cadherin-catenin unit, essential for cell differentiation and maintenance of normal tissue architecture and also plays an important role in Wnt signal transduction pathway. Mutations of CTNNB1 result in stabilisation of a protein that resists degradation, leading to nuclear accumulation of  $\beta$ -catenin. This can be demonstrated by immunostaining and has been described in endometrioid endometrial carcinoma. A number of studies have analysed endometrial carcinomas showing that nuclear accumulation of  $\beta$ -catenin is significantly more common in endometrioid lesions (31–47%) compared with non-endometrioid histology (0–3%).<sup>53</sup>

#### **1.9.3.5 K-ras**

K-ras encodes a small cytoplasmatic, cellular membrane GTPase, which functions as a molecular switch during cell signalling and which is largely related to tumour growth and differentiation. K-ras mutations have been identified in 10–30% of endometrioid endometrial carcinomas but seem to be almost completely absent in serous and clear cell carcinomas of the endometrium.<sup>35 38 125 126</sup> Lagarda et al. and others described to have found more K-ras mutations in MSI-positive carcinomas than in MSI-negative tumours. A possible explanation could be a simultaneous occurrence prior to clonal expansion.<sup>34 138</sup> Endometrial hyperplasia cases and endometrioid endometrial carcinomas were observed to have similar rates of K-ras mutations. This might indicate an early occurrence in endometrial carcinoma development.<sup>139</sup> No relationship has been

found between K-ras mutations and tumour stage, histologic grade, depth of myometrial invasion, age or clinical outcome in endometrioid endometrial carcinomas.<sup>36</sup>

#### **1.9.3.6 PIK3CA**

PIK3CA (Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide) gene locates on chromosome 3q26.32. Phosphatidylinositol-3-kinase (PI3K) is heterodimeric lipid kinase consisting of a catalytic subunit (p110) and a regulatory subunit (p85) in the PI3K/AKT signalling pathway. Mutations in PIK3CA, which codes for the p110a catalytic subunit of PI3K, have been described in various tumours and may contribute to the alteration of the PI3K/AKT signalling pathway in type I endometrial carcinoma.<sup>140-</sup>

<sup>142</sup> Oda et al. described mutations in PIK3CA gene in endometrial carcinomas for the first time. In their series PIK3CA mutations occurred in 36% of the cases, and coexisted frequently with PTEN (15–27%)<sup>35 143</sup> and K-ras mutations<sup>36</sup> suggesting that the PIK3CA mutations cooperate with these alterations in malignant transformation.<sup>144</sup> PIK3CA mutations did not correlate with MSI or  $\beta$ -catenin/CTNNB1 mutations.<sup>145</sup>

Subsequent studies have shown that PIK3CA mutations are frequent in endometrioid endometrial carcinoma, in association with invasion, and adverse prognostic factors such as blood vessel invasion.<sup>142</sup> A number of publications list PIK3CA mutations as a characteristic feature of type I endometrial carcinoma found with a frequency of ca. 30% in contrast to only 5% of type II.<sup>127</sup> However, Hayes et al. found mutations of PIK3CA in 15% and Kuhn et al. in 23,7% of papillary serous carcinoma.<sup>45 146</sup> In fact Konstantinova et al. demonstrated PIK3CA mutations with a high frequency in metastatic tumours and in 50% of samples displaying serous differentiation, serous and mixed endometrioid/serous tumours.<sup>147</sup> Kuhn et al are among the most recent

publications to regard the genetic aberrations involving the PI3K pathway as a major mechanisms in the development of uterine serous carcinoma.<sup>45</sup>

This discrepancy of findings elucidates the early days and difficulty of interpretation of molecular research – in the above case one of the confounding factor is surely the unclear pathologic and molecular differentiation of endometrioid and non-endometrioid tumours.

### **1.9.4 Molecular characteristics of type II endometrial carcinoma**

#### **1.9.4.1 p53**

p53 is a tumour suppressor gene located on the short arm of chromosome 17. Wild type p53 protein contributes to tumour suppression through both arrest of cell proliferation and induction of cell death through apoptosis.<sup>148 149</sup> Inactivation of p53 function through allelic loss, mutation, or complex formation with other nuclear proteins contributes to malignant transformation. This process makes malignant cells more resistant to cytotoxic chemotherapeutic drugs and radiation because of failure in induction of apoptosis.<sup>150</sup> Although p53 is one of the most commonly mutated genes in human tumours<sup>151</sup> the molecular and immunohistochemical investigation of p53 expression in endometrial cancer is contradictory and its role remains to be defined.

Mutations in the p53 gene are a frequent and seemingly characteristic finding in type II serous tumours with positive immunohistochemistry reported in 71–90% of tumours.<sup>123</sup>

<sup>124 45</sup> They are only present in 10–20% of endometrioid endometrial carcinoma, which are mostly high-grade.<sup>145</sup> P53 is regarded the most characteristic genetic alteration of

non-endometrioid endometrial carcinomas<sup>36 37</sup> and may be useful in their distinction from endometrioid endometrial carcinomas.<sup>152</sup>

#### **1.9.4.2 Her-2/neu**

Epidermal growth factor receptor II or Her2/neu is an oncogene that codes for a transmembrane receptor tyrosine kinase involved in cell signalling and located at the long arm of chromosome 17q12.

Endometrial carcinomas are known to sometimes overexpress and/or amplify Her2. Aggressive tumours of high grade or serous histology appear more likely to be Her2 positive.<sup>153 154</sup> However, the exact frequency of Her2 amplification/overexpression in type II endometrial carcinoma remains controversial. Her2 gene amplification has been reported to occur in 6 out of 28 (21%), 17 out of 58 (29%), or 11 out of 26 (42%) of patients with uterine serous papillary cancer<sup>153 155 156</sup>, and Her2 protein overexpression was seen in 12 out of 68 (18%) of cases.<sup>157</sup> In clear cell endometrial cancer Her2 amplification has been described in two out of nine (22%) and three out of six (50%) of the reported cases.<sup>153 155</sup> Overall Her2/neu overexpression or amplification appears to be more frequently found in non-endometrioid endometrial carcinoma (18–80%)<sup>114</sup> than in grade 2 and 3 endometrioid carcinoma (10–30%)<sup>36 37</sup> and has been associated with adverse prognostic parameters including advanced stage, high histological grade, and low overall survival.<sup>114</sup> However, the wide range in reported frequency of Her-2 expression in either type of endometrial cancer has so far rendered its usefulness as a clinical marker controversial. The anti-Her2 monoclonal antibody, trastuzumab, has improved the prognosis of women with Her2-positive breast cancer, both when used as part of adjuvant therapy and in the setting of metastatic disease.<sup>158</sup> In a front-line



chemotherapy trial (GOG protocol #177) the Gynecologic Oncology Group (GOG) analyzed tumours of women with advanced or recurrent endometrial carcinoma, and found that 12% of tumours demonstrated Her2 gene amplification and 20% demonstrated strong (3+) immunohistochemical (IHC) staining for Her2. 21% of grade 3 non-serous tumours and 21% of serous tumours were FISH positive.<sup>155</sup>

The GOG undertook a phase II trial (GOG protocol #181B) of single agent trastuzumab to evaluate its potency against stage III or IV or recurrent Her2-positive (2+ or 3+ immunohistochemical staining) endometrial carcinoma. Of 286 tumours screened 33 (11.5%) were Her2-amplified. 3 of 8 clear (38%) cell carcinomas and 7 of 25 serous carcinomas (28%) screened exhibited Her2 amplification compared with 7% (2/29) of endometrioid adenocarcinomas. Out of the 33 patients who enrolled in the study 18 had tumours with known Her2 amplification. Whilst no major tumour responses were observed, 12 women showed stable disease, 18 had increasing disease, and in 3 cases tumour response was unclear. Neither Her2 overexpression nor Her2 amplification appeared to be associated with progression-free survival or overall survival. The report concluded that trastuzumab as a single agent did not demonstrate activity against endometrial carcinomas with Her2 overexpression or Her2 amplification.<sup>159</sup> So far, whilst Her2 has been reported to be associated with type II endometrial carcinoma, it has not been reliably proven to be of prognostic benefit for poor prognosis endometrial cancer.

#### **1.9.4.3 E-Cadherin**

Cadherins are a family of adhesion molecules essential for tight connection between cells. E-cadherin is a transmembrane protein encoded by CDH1 gene and locates on chromosome 16q22.1. Its extraellular domain regulates cell–cell adhesion via a  $\text{Ca}^{2+}$ -

dependent mechanism. Its role in inhibiting invasion and metastasis is related to its function in prohibiting the first step in the metastatic cascade, namely local invasion.<sup>36</sup>

<sup>37</sup> In vitro analyses have correlated lost or down-regulated expression of E-cadherin in tumour cells with promoting tumour invasion and metastasis in murine models and adverse clinicopathological characteristics in numerous human cancers.<sup>160 161</sup>

Underexpression of E-cadherin is frequent in endometrial carcinoma and may be caused by loss of heterozygosity (LOH) or promoter hypermethylation. LOH at 16q22.1 is seen in almost 60% of non-endometrioid endometrial carcinoma, but in only 22% of endometrioid endometrial carcinoma.<sup>38</sup> In endometrial carcinoma, partial or complete loss of E-cadherin expression correlates with aggressive behaviour.<sup>36</sup>

Kim YT et al. and Mell LK et al. investigated E-Cadherin expression in endometrial cancer in 33 and 102 patients with endometrial carcinoma of various stages, grades and histology types.<sup>162 163</sup> Both studies showed evidence that decreased membranous E-cadherin expression is predictive for endometrial cancer mortality, disease progression, and extrapelvic recurrence, independent of known prognostic factors such as stage, grade, and histological subtype. Both observed an inverse relationship between E-cadherin expression and adverse outcomes. However the prognostic power for type II endometrial cancer has not been reliably shown. On multivariate cox regression analyses in the study of Mell LK et al., a higher E-cadherin expression score was associated with statistically significant decreases in endometrial cancer mortality (HR, 0.23; 95% CI, 0.055– 0.94; P - 0.040), disease progression (HR, 0.28; 95% CI, 0.10– 0.77; P - 0.014), and extrapelvic recurrence (HR, 0.24; 95% CI, 0.062– 0.97; P - 0.045). However, whilst Kim YT et al. showed a significant correlation between aberrant E-

cadherin and type II endometrial cancer (n =5), Mell LK et al. did not find such an association.

Whilst the E-cadherin catenin complex is undoubtedly of enormous relevance for the formation of tight cell-cell adherence, hence cell mobility and growth and thus plays a significant role in tumourigenesis, these are relative downstream products in the signalling cascade towards cell mobility and metastasis. It would be of wider interest to find and influence higher organised proteins which actually manage the above complex.

#### **1.9.4.4 p16**

P16 plays a known role in cell cycle regulation as a tumour suppressor gene located on chromosome 9p21.<sup>37</sup> P16 inactivation can lead to uncontrolled cell growth. This seems to be more frequent in non-endometrioid endometrial carcinoma (40–92%) than in endometrioid endometrial carcinoma (7-25%).<sup>37 53 120</sup> In a population-based study of 316 patients loss of p16 protein expression was significantly related to a subgroup of aggressive endometrial carcinomas and poor prognosis. Thus, absent or minimal nuclear staining was associated with increased age at treatment, higher FIGO stage, serous papillary or clear cell histological types, high histological grade, and aneuploid tumours. Furthermore, there were strong correlations with increased tumour cell proliferation, supporting the role of intact p16 protein as a cell cycle inhibitor.<sup>164</sup>

Loss of p16 expression is also correlated with K-ras and p53 mutations and is associated with high stage, high grade, and poor survival.<sup>36</sup>

However, the importance of the cell cycle regulator p16 is still controversial, since its alteration varies dramatically in several studies, possibly due to the applied techniques and interpretation of staining.<sup>165</sup> Also the underlying mechanism is yet unclear, because neither promoter hypermethylation nor deletion or mutation is frequently found.<sup>164 166</sup>

### **1.9.5 Molecular differentiation and overlapping of endometrioid (type I) and non-endometrioid (type II) endometrial carcinoma**

As much as Bokhman's system of the dualistic model of endometrial cancer helped clinicians recognise the clinical, therapeutic and prognostic gap between type I and II endometrial carcinoma, its oversimplification and classification also poses a risk of misdiagnosing certain types of tumours which either do not fit into the defining criteria of type I and type II endometrial cancer or its clinico-pathological behaviour is crossing the dividing line between the two types.<sup>14</sup>

On the type I side of the division it is particularly the case for grade 3 endometrioid carcinomas. They often take a more aggressive clinical course and have a significantly worse prognosis than grade 1 and 2 endometrial cancers.<sup>167 168</sup>

On the type II side recent publications suggested that the current pathological classification and grading system of high-grade endometrial carcinomas is problematic in both reproducibility and prognostic accuracy.<sup>168 169</sup>

Llobet et al. demonstrated main molecular alterations involved in endometrial carcinoma for endometrioid and non-endometrioid endometrial cancer. Whilst pTEN, MSI, PIK3CA and K-ras mutations are understood to be characteristic features of type I endometrial cancer such mutations only occur in 15-50% of cases. Conversely the same molecular alterations are observed in type II endometrial cancers in up to 15%. Also, whilst p53, E-cadherin and PPP 2R1A mutations are regarded as characteristic for type II endometrial carcinoma they are also detected in grade 3 endometrioid carcinomas and carcinosarcomas. McConnechny et al. suggest that since no single gene mutation or

molecular alteration seems to be sensitive and specific enough to define the risk grouping of these cancers it is likely that the analysis of whole gene panels will be needed to guide subclassification. New sequencing technologies will soon be available to analyse multiple genes and samples simultaneously, making large mutational studies achievable.<sup>52 170</sup>

In their analysis of 152 endometrial tumour tissue samples and 260 endometrial tumour DNA samples McConnechy et al. postulated a 'mutational flow' from 'classic' type I, low grade endometrioid tumours via mixed serous and endometrioid types with mixed clinical aggressiveness to the very aggressive 'classic' uterine papillary serous carcinoma with a predictable mutation profile. In their mutation frequency analysis grade 3 endometrioid cancers were in some cases (p53, PIK3CA and PPP2R1A) based between low grade endometrioid carcinoma (grade 1 and 2) and uterine serous carcinoma. Other results within the same study (pTEN, MSI) could not support the proposed theory of a mutational flow and concurrent clinical pictures of gradual disease development.

However, they demonstrated that the mutation profile of grade 3 endometrioid carcinoma was mostly distinctly different from that of the low grade, endometrioid endometrial carcinoma. These results also demonstrated that more molecular markers are necessary to improve the sub-optimal performance of conventional histopathological assessment of endometrial tumours.<sup>168</sup>

## 1.10 Molecular targeting therapies

There are a number of molecular markers which already have been investigated for their potential role in endometrial cancer. In addition a few agents have been developed trying to target these marker. A variety of agents are currently undergoing clinical development or are already used in trials in endometrial cancer. These agents target tumour cells or the tumour environment including stromal cells, endothelial cells, endothelial precursor cells, pericytes, and immune cells.

In 2010/2011 about 250 clinical trials involving endometrial cancer are registered internationally. 59 of these included some form of targeted therapy. These studies focus almost exclusively on recurrent or persistent disease, none of them focus on type II endometrial cancer. Agents are used as monotherapy or in combination with chemo- or radiotherapy and also with other targeting agents.

Most trials cover three groups of agents: Receptor tyrosine kinase (RTK) -inhibitors or -antibodies, mammalian Target of Rapamycin (mTOR)-inhibitors and oestrogen inhibitors (Selective estrogen receptor modulators (SERMs), aromatase inhibitors). Among the RTK's, the most widely researched molecules are the epidermal growth factor receptor (EGFR) family and the vascular endothelial growth factor – (VEGF) and - receptor (VEGFR) family including well known agents such as Bevacizumab, Trastuzumab and Cetuximab. None of these agents have so far shown any difference in endometrial cancer survival.

A few others trials include agents such as cell cycle inhibitors, mitogen activated protein kinase (MAPK) -inhibition, gonadotropin releasing hormone (GnRH) -agonism, folic acid analogism, histone deacetylase inhibitors (HDAC) -inhibition, the monoclonal

antibody RAV12, CALAA-01 (RNA-interference), UCN-01 (protein kinase C inhibition) and cyclooxygenase (COX II)-inhibition. Again, as yet, these agents have failed to show any significant impact on endometrial cancer survival or played any role as a diagnostic or prognostic tool.

## **1.11 Novel markers used in this research project**

### **1.11.1 Tetraspanin CD151**

Tetraspanins are small membrane proteins expressed in a wide range of species from sponges to mammals, with each organism expressing a large number of tetraspanin family members. Numerous biological processes need tetraspanin input, such as fertilization, inflammation and infection, parasite as well as viral infection, synaptic contacts at neuromuscular junctions, platelet aggregation, maintenance of skin integrity, immune response induction, metastasis suppression as well as tumour progression.<sup>171 172</sup>

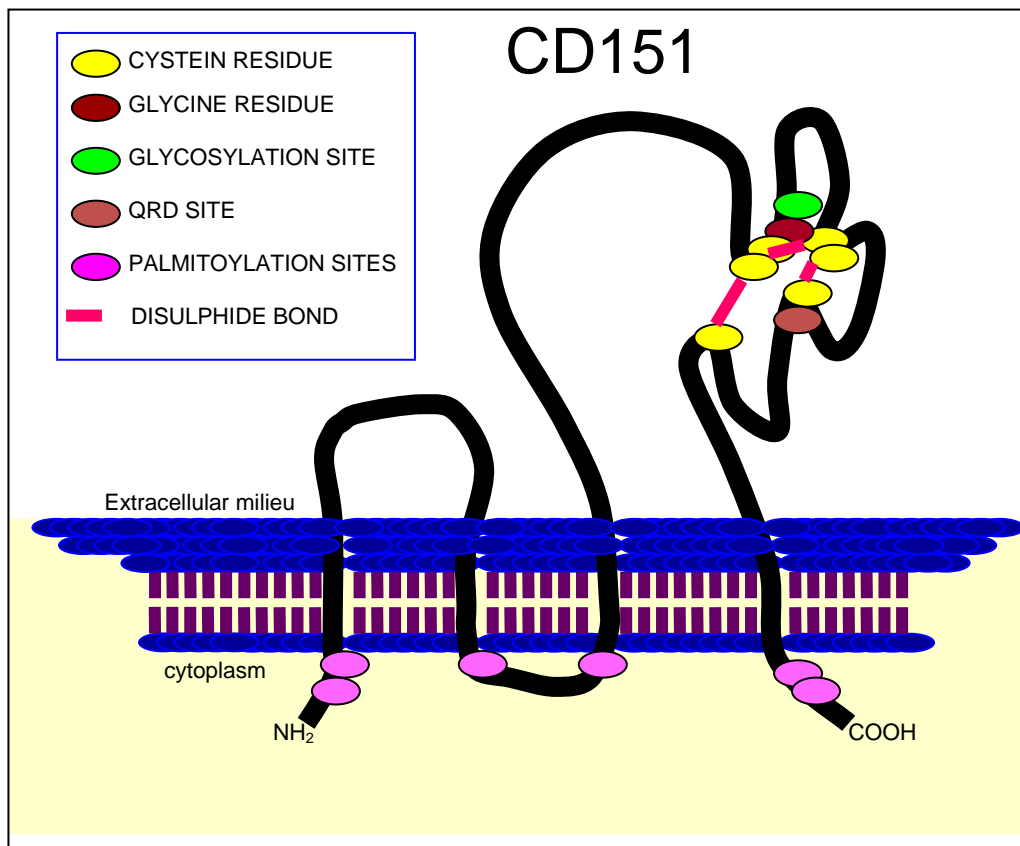
CD151 is one of currently 33 proteins known in the mammalian tetraspanin superfamily.

#### **1.11.1.1 Architectural features of tetraspanins**

A defining feature of the protein family of tetraspanins is its transmembranous architectural structure that crosses the cell membrane four times (Figure 1.4, see page 59). They have short amino- and carboxy-terminal tails, a small intracellular loop between transmembranous region 2 (TM2) and TM3, a small extracellular loop (ECL1) between TM1 and TM2 and a large extracellular loop (ECL2) between TM3 and TM4 (Figure 1.5, see page 61). ECL2 can be subdivided into a mostly constant region and a variable region. The constant region may account for dimerization, the variable region for interactions with non-tetraspanin partner molecules.



**Figure 1.4**

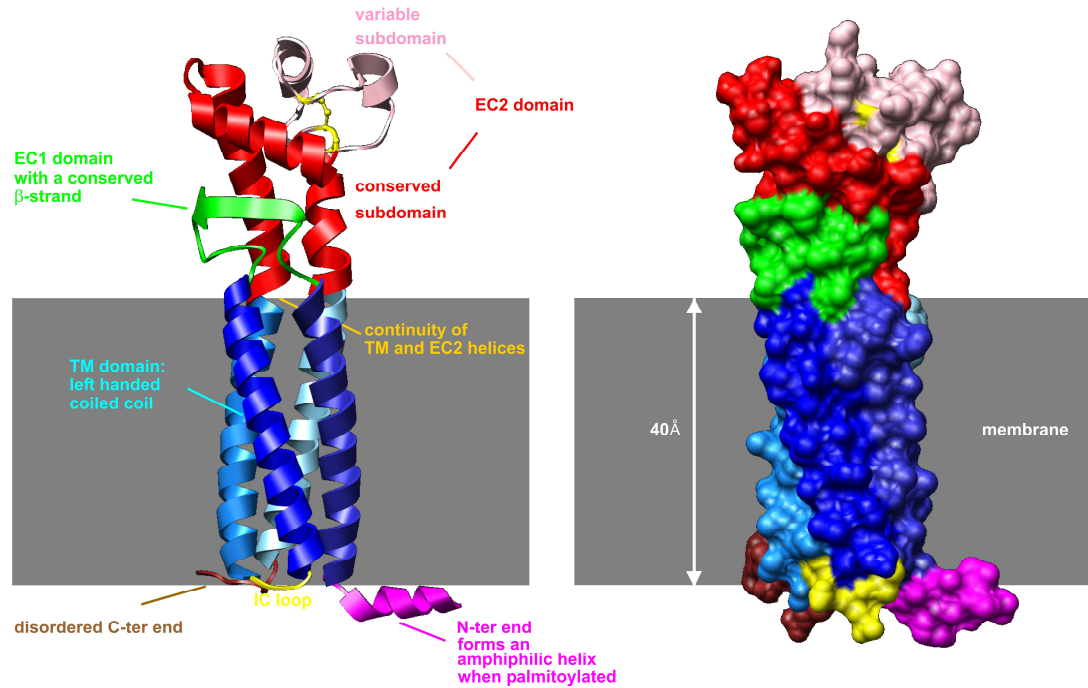


**Transmembranous architectural structure-model of tetraspanin.**  
(kind courtesy of F. Berditchevski, Birmingham, United Kingdom)

#### **1.11.1.2 Locations of tetraspanins**

Apart from the general location in the cell membrane, tetraspanins are also abundant in membranes of various types of endocytic organelles, endosomes and exosomes, 30–100 nm vesicles that are released by many cells. They derive from multivesicular bodies, which either fuse with lysosomes or fuse with the plasma membrane and release their intraluminal vesicles as exosomes. The molecular composition of exosomes reflects their origin from intraluminal vesicles and includes several tetraspanins. Exosomes are thought to constitute a potent mode of intercellular communication that is also important in tumour progression. Thus tetraspanins are able to communicate and travel within the cell with or without endosomes as well as leaving the cell in form of exosomes and multivesicular bodies (MVBs). Tetraspanin-enriched microdomains (TEMs) may appear similar to lipid rafts and indeed have common features. However, they are different structures: They are disrupted by Triton X-100 at 4°C and the signature molecules for classical rafts — glycosylphosphatidylinositol-anchored proteins and caveolin — do not associate with tetraspanins.

**Figure 1.5**



**Predicted 3-dimensional structure of tetraspanin.**

**(kind courtesy of F. Berditchevski, Birmingham, United Kingdom)**

#### **1.11.1.3 Tetraspanin primary interactions**

Tetraspanins play a key role in the formation of complexes called the tetraspanin-enriched microdomains (TEMs), which contain amongst other tetraspanins a number of other transmembrane receptors (e.g. integrins, receptors tyrosine kinases) and thus provide a signalling platform. Laminin-binding integrins particularly  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  are most commonly associated with TEM formation and it has been shown that tetraspanins regulate integrin-dependent adhesion strengthening. Tetraspanins also associate with growth factor receptor, G-protein-coupled receptors and their intracellular associated heterotrimeric G-proteins, several peptidases, transmembrane proteins associated with tumour progression, immunoglobulin superfamily members and cytosolic signal transduction molecules. Palmitoylation of intracellular, juxtamembrane cysteines is thought to be required for initiating tetraspanin–tetraspanin web formation. This also seems to shield tetraspanins from lysosomal lysis and promotes increased cell–cell contact. The formation of the essential tetraspanin enriched microdomain is also partly dependent on the palmitoylation of specific integrins.

#### **1.11.1.4 Core activities of tetraspanins.**

Activities of tetraspanins vary considerably depending on the activation state of the cell as well as the surrounding tissue. Tetraspanins can act either directly, through their laterally associated partner molecules or, less frequently, through ligand binding. In addition, as a major component of exosomes, tetraspanins are likely to be involved in cross-talk between distant cells. According to Maecker et al. in the first review about tetraspanins: “Tetraspanins function via modulating, stabilizing or preventing the

activities of their associated molecules, which vary depending on the TEM composition".<sup>172</sup> Via biosynthesis, compartmentalisation, internalisation and recycling of integrins, or modulating of integrin signalling and trafficking tetraspanins play a key role in cell motility, adhesion, invasion and fusion.<sup>171 173 174</sup>

#### **1.11.1.5 Tetraspanin CD151 and tumour progression**

In vivo tumour cell migration through integrin-dependent pathways is key to the metastatic behaviour of malignant cells. Due to its strong binding properties with laminin binding integrins CD151 is well positioned to modulate integrin-dependent cell spreading, migration, signalling, and adhesion strengthening.<sup>175-177</sup>

Integrin-tetraspanin complexes have been described to be critical for regulating cell invasiveness and controlling intercellular interactions, both of which play a critical role during the metastatic progression.<sup>178</sup> Takeda et al. analysed CD151-null cells and tissue ex-vivo and showed selected alterations in cell outgrowth, migration, aggregation, proliferation, morphology and signalling.<sup>179</sup> Consistent with the close association with laminin-binding integrins they demonstrated that removal of CD151 by antisense, siRNA knockdown or knockout showed selective signalling defects with diminished phosphatidylinositol 3-kinase (PI3K), Akt, and Rac1 pathways and disruption of connections between laminin-binding integrins and at least 5 other proteins which resulted in impaired pathologic angiogenesis. They concluded that CD151 supports pathologic angiogenesis via the influence of laminin-binding integrins on endothelial cells. Furthermore CD151 depletion has been described to either increase<sup>179</sup> or decrease<sup>173 179</sup> cell motility. CD151 has also been shown to promote tumour cell intravasation and subsequent colonization of secondary organs. Blocking CD151 leads to a

significant increase in cell adhesion and a pronounced immobilization of the tumour cell, thus inhibiting migration and preventing cell dissociation and metastasis.<sup>180</sup>

To investigate the role of CD151 in tumour metastasis further, CD151 cDNA was transfected into different tumour cell lines and it was found that these CD151 overexpressing cells were more aggressive than the control cells with enhanced motility and invasion.<sup>181 182</sup> Testa et al. reported that CD151 is involved in an early step in the formation of metastatic foci, such as arrest, extravasation, and/or migration into the connective tissue stroma of the secondary organ.<sup>173</sup>

However, the role of CD151 in proliferation and metastasis of non-hematopoietic cells remains controversial. There were no obvious proliferative defects in CD151-deficient mice and humans.<sup>180 183</sup> Also, deletion of CD151 did not affect proliferation of primary endothelial cells on Matrigel in vitro.<sup>179</sup> Yet Geary et al showed that primary keratinocytes on a laminin substrate were impaired when the cells were CD151 negative.<sup>184</sup> It is possible that CD151 may play a greater role in immunocompromised tumour cells rather than in normal cells.<sup>185</sup> In malignancy CD151 appears to be involved in both - pro-invasive- (rapid migration) and anti-invasive functions (stable cell junctions).<sup>178</sup> Overexpression as well as underexpression of CD151 has been observed in different tumour types. Novitskaya et al. suggested that variations in CD151 effects might be cell-type specific and/or also dependent on the individual host microenvironment.<sup>186</sup> CD151 has been investigated in vivo in a number of cancer types.

Prostate cancer: Ang et al. investigated prostate cancer collecting tissue specimens from 76 primary prostate cancers and 30 benign prostate hyperplasia (BPH) controls.<sup>187</sup> CD151 expression was found to be significantly higher in prostate cancer specimens

compared with BPH specimens ( $p < 0.001$ ). The strongest staining was observed in poorly differentiated tumour specimen, whereas well differentiated cancers expressed the weakest staining for CD151 ( $p < 0.001$ ). CD151 expression showed a negative correlation to overall survival, i.e. higher levels of CD151 were associated with poorer prognosis. This effect was independent of the patients' age or preoperative prostate-specific antigen values and superior in the predictive ability of the Gleason score. They concluded that CD151 had better predicting value for the clinical outcome of prostate cancer patients than does the traditional histologic grading method (Gleason grading).

Lung cancer: Non small cell lung cancer was investigated by Tokuhara et al. using reverse transcription-PCR and immunohistochemistry.<sup>188</sup> Whereas 86 out of the 146 collected tumour specimens were positive for the CD151 gene, 59 had tumours that were negative for the CD151 gene. The overall survival rate of patients with CD151-positive tumours was lower than that of CD151-negative patients (51.9% versus 73.1%;  $p = 0.013$ ). They concluded that high CD151 gene expression in lung cancer may be associated with a poor prognosis.

Breast cancer: Yang et al. postulated an acceleration of growth caused by CD151 regulating  $\alpha 6$  integrin activity.<sup>189</sup> They observed an increased CD151 expression in a subset of human breast cancer samples, particularly those of high grade and/or triple negative tumours – oestrogen receptor (ER) negative, progesterone receptor (PR) negative and human epidermal growth factor receptor 2 (Her 2) negative – which appeared to be a significant marker for poor outcome. In this study CD151 ablation markedly reduced migration, invasion, spreading, and signalling of basal-like mammary tumour cells and showed signs of disrupted epidermal growth factor receptor (EGFR)- $\alpha 6$ - integrin collaboration. In a basal-like mammary tumour cell line, they demonstrated

that CD151 ablation delayed tumour progression. They concluded that CD151 is a potential therapeutic target with relative selectivity (compared with laminin-binding integrins) for pathologic rather than normal physiology.

Colorectal cancer: Interestingly Chien et al. demonstrated very different results.<sup>190</sup> They investigated a cohort of 137 paired cases of colorectal carcinoma and normal colon tissues examined by immunohistochemical staining and western blot for the expression of CD151. They showed that expression of CD151 protein was reduced in the colon cancer tissues compared with the surrounding normal tissues. One explanation was the observation that the expression of CD151 was negatively regulated by hypoxia inducible factor-1- dependent hypoxic stress. Suppression of CD151 by hypoxia caused the detachment of cancer cells from the surrounding matrix and neighbouring cells whereas restoration of CD151 expression during re-oxygenation facilitated the adhesion capacity.

Urothelial Carcinoma: Recently Minner et al. published data from a tissue microarray with 664 tumour specimen. Like Chien et al. they observed an association between decreased levels of CD151 and advanced tumour stage and high grade in urothelial bladder cancer.<sup>191</sup>

Given the above findings of CD151's potential central role in cell motility and metastasis, its' clinical behaviour in hormonally dependent tissues such as breast cancer, as well as its' so far controversial effects observed, CD151 was found to be a highly interesting and suitable marker for investigation of poor prognosis endometrial tumours in which tetraspanin activity has never been investigated.



### 1.11.2 Clusterin

Clusterin is a glycoprotein that has a nearly ubiquitous tissue and fluid distribution, found amongst others in breast, prostate and kidney.<sup>192</sup> It was previously also known as „apolipoprotein J”, “testosterone-repressed prostate message-2”, and “sulfated glycolprotein-2”. Located on chromosome 8p21-p12 Clusterin (CLU) is coded by a single nine-exon gene spanning 17kb. The gene encodes 3 CLU isoforms, and differences in subcellular localisation give direction to the understanding of their function.<sup>193</sup>

CLU has been implicated in various cell functions including apoptotic cell death, cell cycle regulation, cell adhesion, tissue remodelling, and immune system regulation involved in physiologic or pathologic processes such as carcinogenesis and tumour progression.<sup>194-196</sup> Such an expression pattern is pointing at a potential key role in cellular homeostasis, and the protein is known to have both pro-apoptotic and anti-apoptotic functions.

#### 1.11.2.1 Clusterin isoforms

This functional dichotomy may be related to at least three isoforms, two of which are translated into a secreted, cytoplasmic 80kDa (glycosylated, proteolytically cleaved) and 60 kDa (non-glycosylated) clusterin protein (sCLU), and one of which is alternately spliced and translated into a nuclear clusterin protein (nCLU, ~ 55 kDa). Reacting to various types of cell damage, nCLU is released inducing apoptosis<sup>197</sup>, whilst sCLU (75–80 kDa) seems to act as an anti-apoptotic/pro-survival or a proliferative protein in certain cancer cells.<sup>198 199</sup>

#### **1.11.2.2 Clusterin and malignancy**

A multitude of data has been generated about the roles of clusterin in malignant disease with seemingly conflicting information indicating in some cancers an oncogenic or cancer-cell-protective activity<sup>200</sup> and involvement in tumorigenesis while in other cancers data are suggestive of a tumour suppressor role. Furthermore, studies have observed decreased as well as increased levels of expression within the same tumour entity.<sup>197 201 202</sup>

#### **1.11.2.3 Clusterin as an oncogene**

A number of studies have shown significant overexpression of CLU in disease and normal tissue, in animal models and cell lines indicating an oncogenic role. Furthermore increased expression correlates with disease severity in a number of cases. In colon<sup>203</sup><sup>204</sup> ovarian<sup>205 206</sup>, breast<sup>207 208</sup>, endometrial<sup>209</sup> and bladder cancer<sup>210</sup> clusterin overexpression was associated with disease progression, recurrence and survival. In renal cancer multivariate analyses revealed that strong expression of clusterin was an independent predictor of tumour recurrence and overall survival.<sup>211</sup> In hepatocellular carcinoma univariate analysis showed recurrence free survival was significantly lower in patients with strong clusterin expression.<sup>212</sup> In cervical cancer Watari et al demonstrated in his cohort of patients who all underwent systematic lymphadenectomy and radical surgery that CLU positivity was an independent prognostic marker for advanced disease.<sup>213</sup>

#### **1.11.2.4 Clusterin as a tumour suppressor**

There is also a variety of publications describing underexpression or downregulation of CLU in tumourigenesis indicating a potential role as a tumour suppressor gene.

In prostate cancer such a possible role has been suggested by Caporali et al.<sup>214</sup> They found that in transgenic adenocarcinoma mouse prostate (TRAMP) mice which spontaneously develop prostate cancer, CLU was downregulated during prostate cancer onset and progression. Such a role is supported by Rauhala et al. who found clusterin expression to be significantly reduced in untreated and hormone-refractory prostate carcinomas.<sup>201</sup>

In colon cancer cell lines Chen et al found that transient transfection of clusterin directly enhanced basal and chemotherapy-induced apoptosis. Furthermore clusterin-induced apoptosis was inhibited by antisense clusterin.<sup>215</sup> In fine needle aspirates of pancreatic adenocarcinoma Jhala et al. found clusterin staining significantly less frequently than in normal reactive ductal epithelium.<sup>216</sup>

However, some studies have found that expression may not necessarily be related to disease progression or tumour size.<sup>217 216 218</sup>

#### **1.11.2.5 Clusterin isoforms in malignancies**

Contradictions and alternative hypotheses still exist about the various roles of clusterin, and researchers are still unsure whether CLU is now a positive or a negative modulator of mammalian tumourigenesis.<sup>219</sup> The likely answer is that it is able to function in both ways.

This might be possible and explained through the different isoforms as well as understanding and discerning the changing biological requirements of a tumour in its course over time, from its early development through to survival under the pressures of environment, hormones, chemotherapy etc. As an example Pucci et al found clusterin in a nuclear localization in healthy colonic mucosa being consistent with the involvement of the proapoptotic nuclear clusterin form in the regulation of cell cycle progression and in cell death induction.<sup>220</sup> Progression towards tumour and particularly towards high-grade and metastatic carcinoma saw clusterin being distributed to the cytoplasm. Protein extracts from such cells documented the complete loss of the pro-apoptotic nuclear form and a cytoplasmic overexpression of the highly glycosylated anti-apoptotic form. These data suggest that the controversial data on clusterin function in tumours may be in part related to the pattern shift of its isoform production.<sup>220</sup>

#### **1.11.2.6 Clusterin in targeted therapy**

Antisense oligonucleotide (ASO) therapy against clusterin is currently used in clinical trials to evaluate its efficacy in improving androgen deprivation therapy and/or prevent chemoresistance.<sup>221 222</sup> Clusterin has also been suggested to be a potential stool biomarker for colon cancer screening.<sup>223</sup> In breast cancer, results from a phase II trial where clusterin ASO therapy was added to a taxane chemotherapy, showed no increase in treatment response.<sup>224</sup>

#### **1.11.2.7 Clusterin and endometrial carcinoma**

In endometrial carcinoma Ahn H.J. et al. found that sCLU was overexpressed in endometrioid endometrial carcinoma (EEC) but not in papillary serous type cancers compared to normal endometrium.<sup>209</sup> The biological role of over-expression of sCLU in the endometrioid carcinoma might be anti-apoptotic or pro-survival, similar to the situation in other carcinomas.

In view of the central role of CLU in tumourigenesis and the urgent need for molecular factors to distinguish the two phenotypes of endometrial carcinomas, Clusterin was chosen as one of the molecular markers to investigate in the present study.

## 1.12 Hypothesis

This study was set out to answer the following hypotheses:

1. Based on clinical observations we hypothesise that G3 endometrioid endometrial carcinoma belongs to a group of poor outcome uterine malignancies which fulfills more criteria of type II rather than type I endometrial carcinoma.
2. Based on the findings in breast-, prostate- and colon carcinoma we hypothesise that CD151 is a useful prognostic marker for poor prognosis endometrial carcinoma.
3. Based on the findings in breast-, prostate-, ovarian- and colon carcinoma we hypothesise that Clusterin is a useful prognostic marker for poor prognosis endometrial carcinoma.
4. Investigating the different groups within poor prognosis endometrial cancers we hypothesise that CD151 and/or Clusterin will provide new information to characterise these groups.

## **1.13 Aims of the thesis**

1. To compare immunohistochemistry profiles and survival outcome of grade 3 endometrioid cancers with type 2 cancers of the cohort investigated.
2. To assess the prognostic significance of CD151
3. To assess the prognostic significance of Clusterin
4. To compare the expression profiles of CD151 and Clusterin with the clinical and pathological features of the cohort investigated.
5. To compare the expression profiles of CD151 and Clusterin with the expression profiles of oestrogen receptor (ER), progesterone receptor (PR), p53 and Her-2 of the cohort investigated.

## **CHAPTER**

# **2 MATERIAL AND METHODS**



## **2.1 Setting**

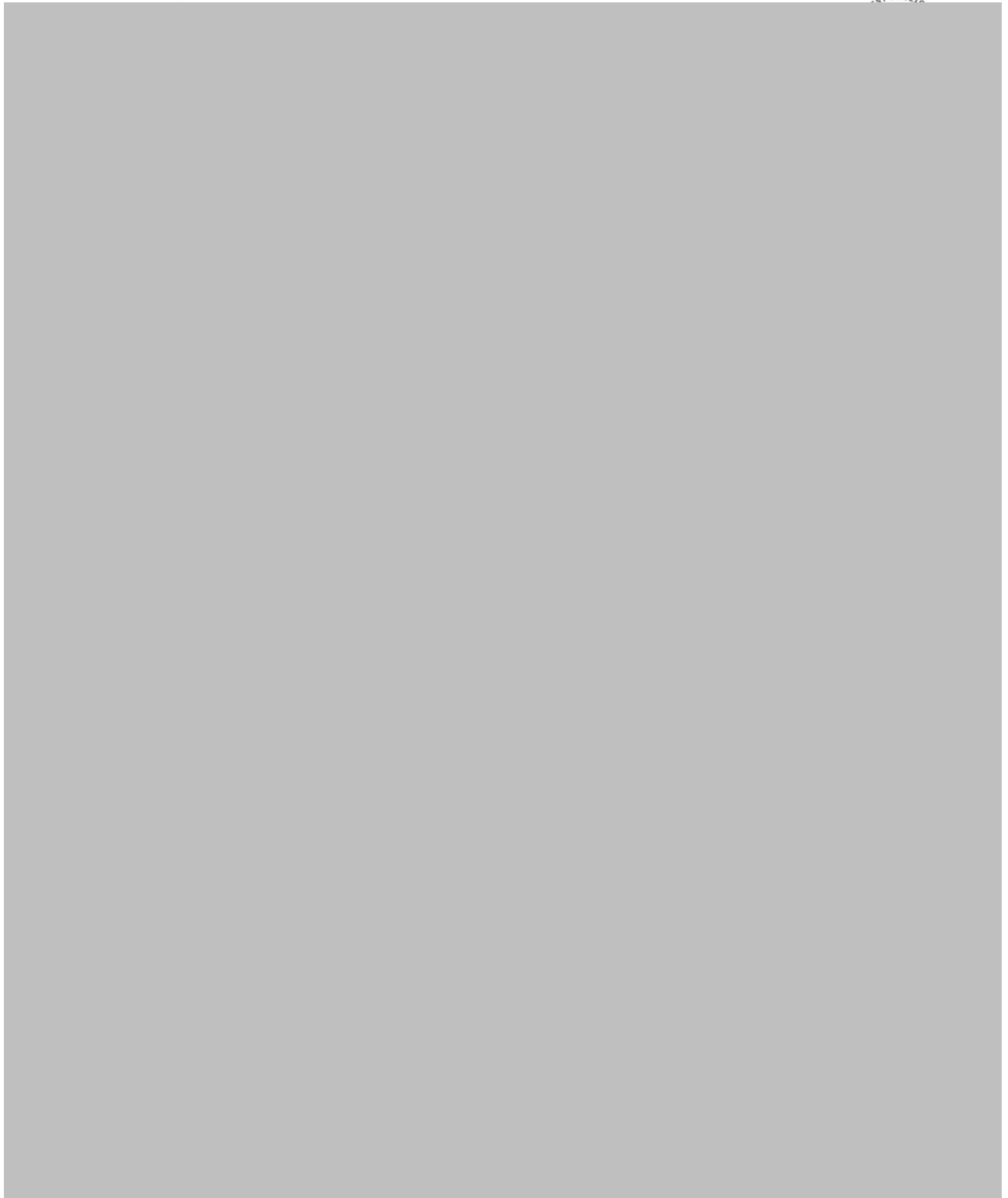
Gloucestershire Hospitals NHS Foundation Trust (GHNHSFT), Gloucestershire, United Kingdom. All patients in this study were seen and treated in Cheltenham General Hospital and/or Gloucestershire Royal Hospital, Gloucester.

## **2.2 Patients and Specimen characteristics**

Upon approval from the Southmead research ethics committee (REC ref.07/H0102/64), archived formalin-fixed and paraffin-embedded tissue blocks and corresponding pathology reports were collected from all patients who underwent treatment for International Federation of Gynaecology and Obstetrics (FIGO) stage IA–IV uterine cancers at the Gloucestershire NHS Foundation Trust Hospitals between 01.01.1997 and 31.12.2002. Histology types grade 3 endometrioid endometrial adenocarcinoma (G3 EEC), uterine papillary serous carcinoma (UPSC), clear cell carcinoma (CC), carcinosarcoma (MMMT), sarcoma and malignant uterine epithelial tumours of mixed histology were selected. Architectural grading was based on the degree of glandular differentiation in accordance with the FIGO guidelines.

Clinicopathological information and survival data were abstracted from an ongoing regional prospectively collected clinical database held at the South West Public Health Observatory (SWPHO), Bristol, United Kingdom.

**Figure 2.1**



**South West Public Health Observatory (SWPHO) data sheet for endometrial cancer by the South West Cancer Intelligence Unit.**

The information in this database is gathered by standardized data forms which have to be completed at the point a certain diagnose has been made and a treatment regimen commenced (Figure 2.1, see page 76).

This form is designed for all patients with endometrial cancer and routinely completed by a member of the gynaecology-oncology team of the GHNHSFT. The purpose and benefit of the SWPHO data sheet is to provide detailed information on how each individual patient has been surgically and systemically treated. It is divided into three sections: surgery, histopathology and oncology.

The surgical section comprises details on the date of operation, the fact whether surgery has been conducted as part of a clinical trial and whether it is a case of primary disease or tumour progress or recurrence. The name of the surgeon and his/her level of training is disclosed and details given on the surgical approach such as the way of incision or via laparoscopy, clinical findings regarding the extent of disease and organs involved as well as details of tissue and/or organs removed. The histopathology section renders information such as the histology reference number and date of reporting, comprises histological typing, grading, detailed description of involved organs such as depth of endometrium invaded, parametrial, ovarian, cervical and vaginal involvement, number and region of lymphnodes sampled and their tumour involvement, metastasation to distant organs, the cytology of peritoneal washings and the final TNM and FIGO stage. The oncology section comprises details on adjuvant therapy such as radio- or chemotherapy. The oncology number is disclosed and information given whether treatment has been subject to a clinical trial. The intention of treatment is stated – whether radical, curative or palliative. In the case of radiotherapy treatment the regimes of external beam and/or brachytherapy are outlined in view of site and size, number of

fractions, the total amount of radiation used measured in gray (Gy) and the overall treatment time. In the case of chemotherapy it is stated whether the intention and timing of treatment was concomitant, adjuvant or palliative, the number of cycles of the specific regime used and information is given on the extent of the response under treatment – complete or partial regression (CR/PR), stable or progressive disease (SD/PD).

All patients were kept under regular surveillance for 5 years, follow-up details for longer than five years were abstracted from hospital records from other attendances.

The survival data used in this study are believed to be very robust due to the centralized computed documentation system covering a whole county. As long as a patient was resident in the county, all hospital attendances throughout the entire region would be documented in the GHNHSFT computer system. Additionally, every single blood sample obtained in one of the NHS facilities or in any GP surgery in the region would be visible via this system and thus proof at least that the patient in question was alive at the given date and where she was last seen. Additionally, counter-checking all patients in the in-house database of the GHNHSFT Oncology Services provided extra safety particularly by identifying recurrence/tumour progression data. Clinical endpoints were survival or death of disease or other causes or date of recurrence as recorded in the hospital notes. Data on death and cause of death was cross referenced against cancer registry data to ensure accuracy.

## 2.3 The tissue microarray

Tissue microarrays (TMAs) consist of paraffin blocks where up to several thousand individual tissue cores have been inserted in an arrayed fashion. They are produced by a method of serially re-locating tissue from many conventional histology paraffin blocks into a single new block and then cut into fine sections such that tissue samples from multiple patients or donor specimen are arrayed on a single microscopic slide to be assayed and visualised simultaneously.

This is done by using a hollow needle attached to a microarrayer to biopsy standard histology sections and placing the core into a precise position in an array on a recipient paraffin block. The microarrayer provides repetitive and precise positioning of the tissue-cores up to a variance of less than 0.1 mm. This technique, originally described by Wan et al in 1987 in the Journal of Immunological Methods was a modification of Battifora's "sausage" block technique where tissue cores were placed in specific pre-formed slots in a block.<sup>225 226</sup> The technique gained international attention when Kononen and colleagues in the laboratory of Ollie Kallioniemi published it in Nature Medicine in 1998.<sup>227</sup>

These miniaturized collections of tissue spots result in a significant increase in throughput for in situ examination of gene status or protein expression from archival specimens. The simplification of specimen handling and processing enables researchers to extend their cohorts exponentially which improves the quality and significance of the findings about the biomarker investigated. Processing quality is further improved by the fact that all specimens are arrayed on a single slide which means staining conditions are identical and consistent with precisely the same antibody dilution. Additionally, the

possibility of looking at a series of biomarkers simultaneously in exactly the same specimen, time and circumstances again improves quality and reliability.

All available methods for examination of histological sections, including immunohistochemistry, in situ hybridization, DNA ploidy analysis, nuclear morphometry, and fluorescent in situ hybridization can be performed on sections from TMA blocks. Many clinical specimens contain limited tissue for study. TMAs can expand the number of studies that can be performed from either limited or extremely valuable specimens. TMAs have been validated as useful tools for the evaluation of various biomarkers, especially in oncology patients.

## **2.4 Tissue microarray construction**

Tissue microarray construction requires detailed planning of successive basic steps which are outlined in accordance with recommendations recently summarised by Professor Manuel Salto-Tellez, Chair of Molecular Pathology at Queens University Belfast presented at the Biomarkers, Biostatistics and Novel Clinical Trial Design workshop held in September 2012 in Nottingham. (<http://ncrndev.org.uk/downloads/BI%20CSG%20Workshops/TMA/Salto-Tellez.pdf>).

Listed below are the 10 recommended basic steps of tissue microarray construction whilst each step is described in detail if required within the following chapters.

1. Collection of the pathology files of the cases included in the study cohort.
2. Retrieval of the histology slides, review of histology and marking out representative areas on a H/E stained slide by a pathologist.
3. Retrieval of paraffin blocks.

4. Marking out areas to be punched on paraffin blocks using H/E slide as guide.
5. Selection of punch size and recipient block.
6. Preparation of tissue microarray map.
7. Punching out tissue cores and transfer to recipient block.
8. Cut H/E slide from arrayed block
9. Review of histology
10. Repair of array block by replacing non-representative tissue cores

#### **2.4.1 Selection of tumour blocks**

A pathologist with a special interest in gynaecologic pathology reviewed all hematoxylin- and eosin-stained (H&E) slides of each tumour to confirm the original diagnosis of endometrioid, uterine serous papillary or clear cell endometrial cancer, sarcoma, carcinosarcoma or mixed histology. Corresponding paraffin blocks were then obtained from the tissue archives. A representative paraffin-embedded tumour block was selected after review of H&E- sections and two separate areas of tumour were marked on the slide and corresponding tissue block. As the block might contain more than one tissue diagnosis of interest for obtaining tissue cores for a TMA, each circled tissue diagnosis on the slide was also assigned a number. (Figure 2.2, see page 82)

**Figure 2.2**



**Paraffin embedded tumour tissue blocks.**

### **2.4.2 Design and mapping**

The design and planning of the tissue microarray construction was carefully conducted to facilitate the following requirements:

1. Logical and successive collection of tissue specimens to avoid missing or duplication of cases.
2. Design of a basic system which would be repeatable for each array-block yet render every block, and thus slide, individually recognisable at a glance.
3. Precise documentation and mapping system of every single position of tissue specimens to render the localisation of each individual case in any given sector of the block swiftly and failsafe and to
4. Aid visual detection of differing slides and specimens in the same assignment.



The basic metric dimensions required for a tissue microarray paraffin block are such that its sections fit on a standard microscope glass slide of 25x75mm. The use of standard microscope glass slides of 25x75mm as carriers of the sectioned tissue microarray specimen is advisable to ensure routine processing, quick and safe throughput, multiple use by various people and institutions as well as storage and postage. To be safely placed on the standard microscope glass slide the mounting paraffin block for the tissue microarray is approximately 20 x 45 mm in dimension.

The standard size of a single tissue core in a tissue microarray core is 0.4 to 1 mm in diameter. Cores can be placed as close to each other in the paraffin block as the physical practicality and precision of an arrayer machine allows. However, in order to retain the stability of the paraffin structure surrounding the tissue cores as well as visibility and ability to keep them separate during the processing of the blocks and slides, it is advisable to space the cores 0.5 to 2 mm apart.

For this study it was calculated to fit 6 to 8 rows of tissue specimen into one paraffin block, each row consisting of 10 to 15 tissue cores, each core 0.6mm in diameter and 1 mm apart from any given neighbour. Thus a single paraffin block would contain up to 120 tissue cores. Given 156 tumour cases with at least two samples each plus a certain number of control samples it was estimated to require space for 400-500 tissue cores. Therefore, it was decided to produce 5 tissue microarrays in 20 x 45 mm paraffin blocks.

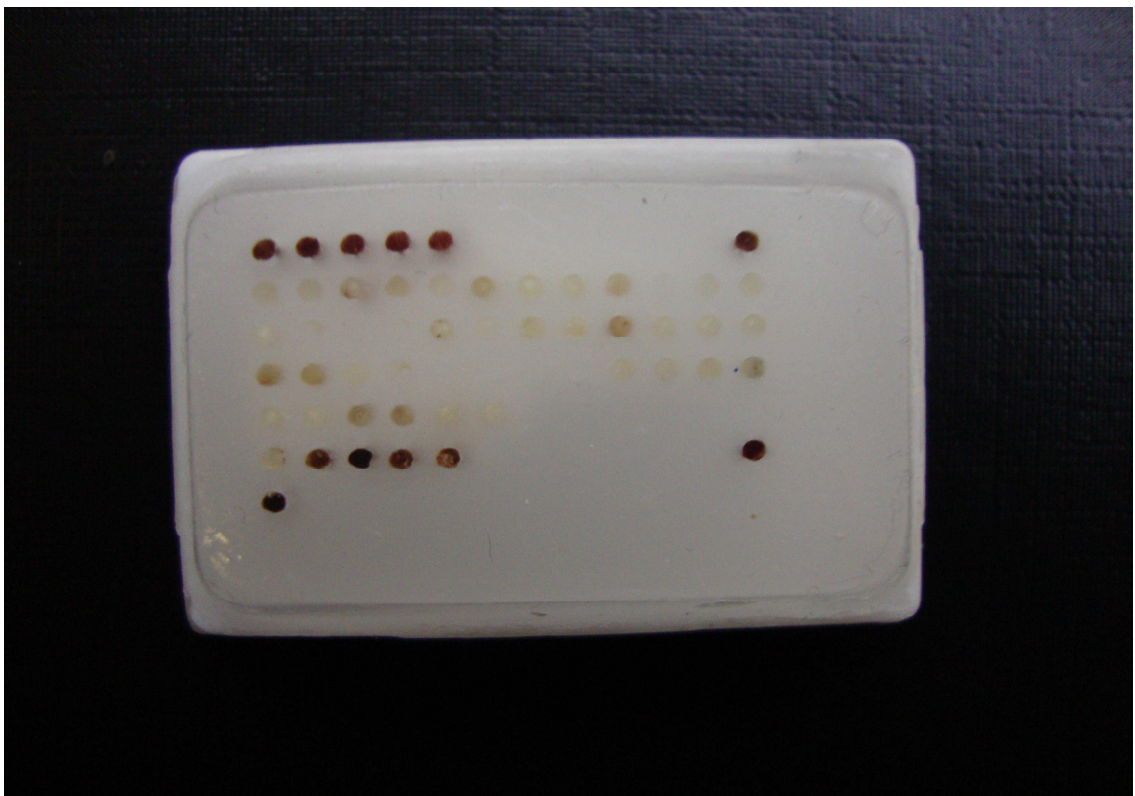
#### **2.4.2.1 Tissue microarray block and slide identification system**

Having more than 1 tissue microarray with each of them containing specimens all of which should undergo exactly the same number and type of experiments, an identification system is required with the ability to differentiate the microarrays from each other and recognise them individually and immediately at a glance. In the case of this study it was necessary to devise a system to identify and differentiate 5 tissue microarray blocks and respective slides and also be able to retain their order at all times. Figure 2.3 (see page 85) depicts an exemplary microarray constructed for this study demonstrating the arrangement and pattern of tissue cores.

1. There are 12 core positions (i.e. positions to receive tissue cores) to each row with a maximum of 7 rows per slide.
2. The top and bottom row (row 1 and 7) are the “block-identification-rows” with at least one core at either end (position 1 and 12).
3. Positions 1 to 5 in the top and bottom row are filled with placental tissue control cores according to the number of the tissue microarray block. Thus, “TMA Block 1” has a tissue core at position 1 and 12, “TMA Block 2” has tissue cores at position 1 and 2 and 12, “TMA Block 3” has tissue cores at position 1-3 and 12 etc. The number of cores in the top and bottom row on the left side is well visible with the naked eye and renders them instantaneously identifiable within the 5 blocks of TMAs of this study.
4. In the very bottom left corner, below position 1 of row 6, a single tissue core is placed to avoid any chance of disorientation on the future TMA slide due to

handling errors of a slide. This core ensures the correct position/orientation the glass slide is held once a TMA is sliced up and placed on a glass mount. If the slide is inadvertently turned up-side down or sideways, this single core would be found (falsely) either above row 1 or below position 12 of row 6, thus the slide be recognised as wrongly orientated or held.

**Figure 2.3**



**TMA block 5 – Exemplary paraffin block with tissue cores embedded.**

#### **2.4.2.2 Tissue core identification and mapping system**

An essential requirement for the TMA is a robust link between each core to its original donor tissue and to the patient's anonymized clinical dataset. A code was developed identifying each tissue core in the TMA encoding the following information:

1. The first number refers to the position of the tumour in the database
2. The double letters refer to the initials of the patient's name.
3. The 6 digit number refers to the year of collection and the histology number of the tumour.
4. The last letter refers to the duplicate number of this tissue specimen, i.e. A or B.

Example: 12-LE-97 3898 A. Given the slide identification system, explained above, only row 2 to 5 in the TMA blocks contained tumour specimen of the study cohort. All other positions were filled with control tissue. Figure 2.4 (see page 87) depicts the specimen reference spreadsheet identifying every position of each tissue core in the five TMA blocks. Each Excel spreadsheet maps out the pattern and position of every tissue core at a glance.

**Figure 2.4**

control											control
2-HV-97 5659	2-HV-97 5659	3-EV-97 5361	3-EV-97 5361	5-TJ-97 1079	5-TJ-97 1079	7-HK-97 4329	7-HK-97 4329	8-BM-97 8597	8-BM-97 8597	9-RS-97 8239	9-RS-97 8239
A	B	A	B	A	B	A	B	A	B	A	B
10-NM-97 8988	10-NM-97 8988	11-WJ-97 14774	11-WJ-97 14774	12-LE-97 3898	12-LE-97 3898	13-GA-97 4764	13-GA-97 4764	14-MD-97 14145	14-MD-97 14145	15-ES-97 16017	15-ES-97 16017
A	B	A	B	A	B	A	B	A	B	A	B
16-HG-98 7569/70	16-HG-98 7569/70	17-OK-98 6256/7	17-OK-98 6256/7	18-KJ-98 7572	18-KJ-98 7572	19-CM-98 1412	19-CM-98 1412	20-PM-h19809849	20-PM-h19809849	21-HB-h19808752	21-HB-h19808752
A	B	A	B	A	B	A	B	A	B	A	B
23-WC-98 7726	23-WC-98 7726	24-JD-h9814398	24-JD-h9814398	25-ME-h19813062	25-ME-h19813062	26-PM-h19815277	26-PM-h19815277	27-WB-99 3039	27-WB-99 3039	28-BJ-h19906145	28-BJ-h19906145
A	B	A	B	A	B	A	B	A	B	A	B
29-CM-99 03781	29-CM-99 03781	30-DD-99 6896	30-DD-99 6896	31-BB-h19909114	31-BB-h19909114	32-LK-h19904777	32-LK-h19904777	33-BV-99 05434	33-BV-99 05434	34-LL-h19979498	34-LL-h19979498
A	B	A	B	A	B	A	B	A	B	A	B
control											control
control											control

0,6 mm CORE, 2mm GAP

BLOCK 1 EmCA 2-34

**Tissue core identification and mapping system.** The Excel spreadsheet maps out the view on top of TMA block 1. Each study tissue core has an individual code consisting of a number followed by two letters and again followed by a number each separated by a hyphen. The first number refers to the position of the tumour in the database. The double letters refer to the initials of the patient's name. The 6 digit number refers to the year of collection and the histology number of the tumour. Sometimes the last number is headed by the letter h which is identifying the histology number of the tissue instead. A capital letter in the box below the code refers to the duplicate number of this tissue specimen, i.e. A or B.

### **2.4.3 Instrumentation and equipment set-up**

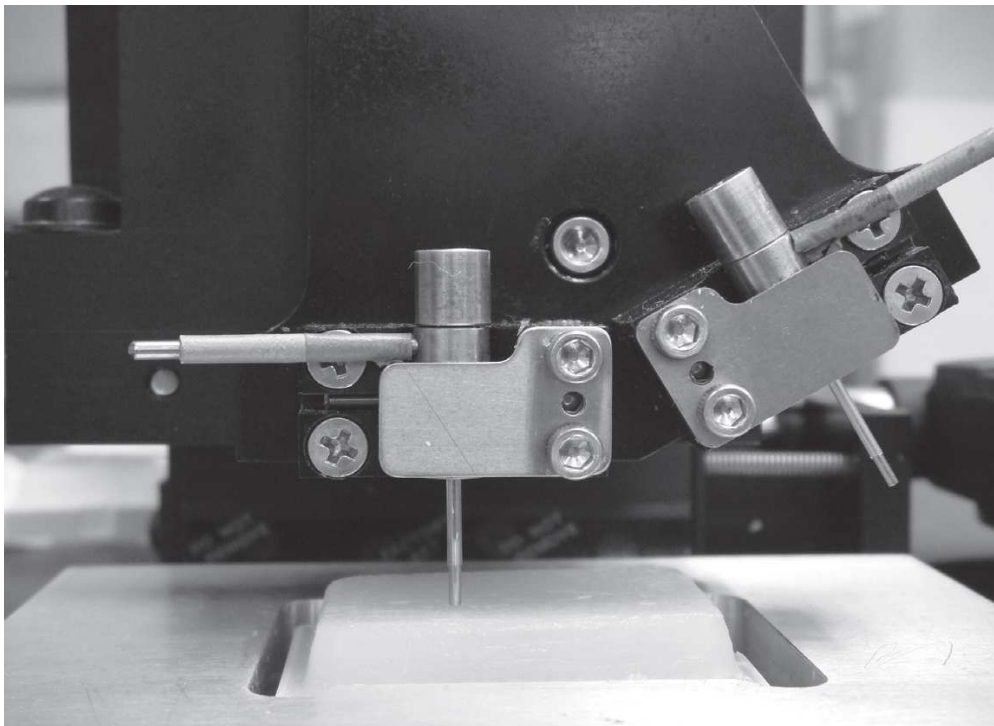
A simple, manually operated tissue microarrayer by Beecher Instruments (Model: Manual Tissue Microarrayer MTA-1) was used. (Figure 2.5, see below). The central part is a rotating turret with two punch needles mounted on it:

Needle 1: To punch a regular array of holes in a recipient block.

Needle 2: To extract cores from marked regions of donor tissue blocks and inject these cores into the holes of the recipient block.

Needle 2 is slightly thinner than needle 1 so that the content of needle 2 fits firmly into the pre-formed hole. X-Y micrometers define the coordinates of the array and its micrometer adjustment knobs (Figure 2.6, see page 89) facilitate adjustment of cored tissues in straight rows, easily visualised in the array setup.

**Figure 2.5**



**Mounted punch needles on tissue microarrayer by Beecher Instruments.**

Before the arraying process begins each donor tissue block and corresponding slide pair are arranged in the order that they will be used following the map design. Prior to placement in the arrayer the recipient blocks are “faced off” on both front and back sides using a rotary microtome to ensure that the block face is smooth and all arrays made will be in the identical plane. This minimizes the amount of block realignment that will be necessary during sectioning and helps to optimize the number of complete sections that an array block will yield. The recipient block is then placed into the base of the arrayer.

**Figure 2.6**



**Manual Tissue Microarrayer MTA-1 by Beecher Instruments with visible adjustment manuals for precise movement across the paraffin block.**

To ensure the alignment of the punch needles, the recipient needle (needle 2) is moved into position and makes a mark in the paraffin. This is then repeated for the donor needle (needle 1) so that both marks are precisely at the same position. The needles are moved to the position of the first punch with the x- and y-axis micrometer adjustment knobs. The position of the punches over the block is assessed by gently pushing down on them until a mark is made in the paraffin, making adjustments with the micrometer knobs until the desired position is attained. Then the micrometer is zeroed for the X and Y axis. At last the “depth stop” is adjusted, which determines how deep the punch will be forced into the recipient block. Only the depth of the recipient core can be set by the depth stop. There is no depth stop to determine or control the depth of the donor core due to the varying thickness of the donor tissue block, which will naturally determine the limit of the depth or length of the cores that can be taken. It is important to choose tumour tissue blocks of appropriate depth for the depth of the thinnest block will determine the maximum thickness of the TMA and dictate the maximum available sections cut from this TMA block. The arrayer is then aligned and ready to be used for one specific block.

#### **2.4.4 Tissue microarray construction**

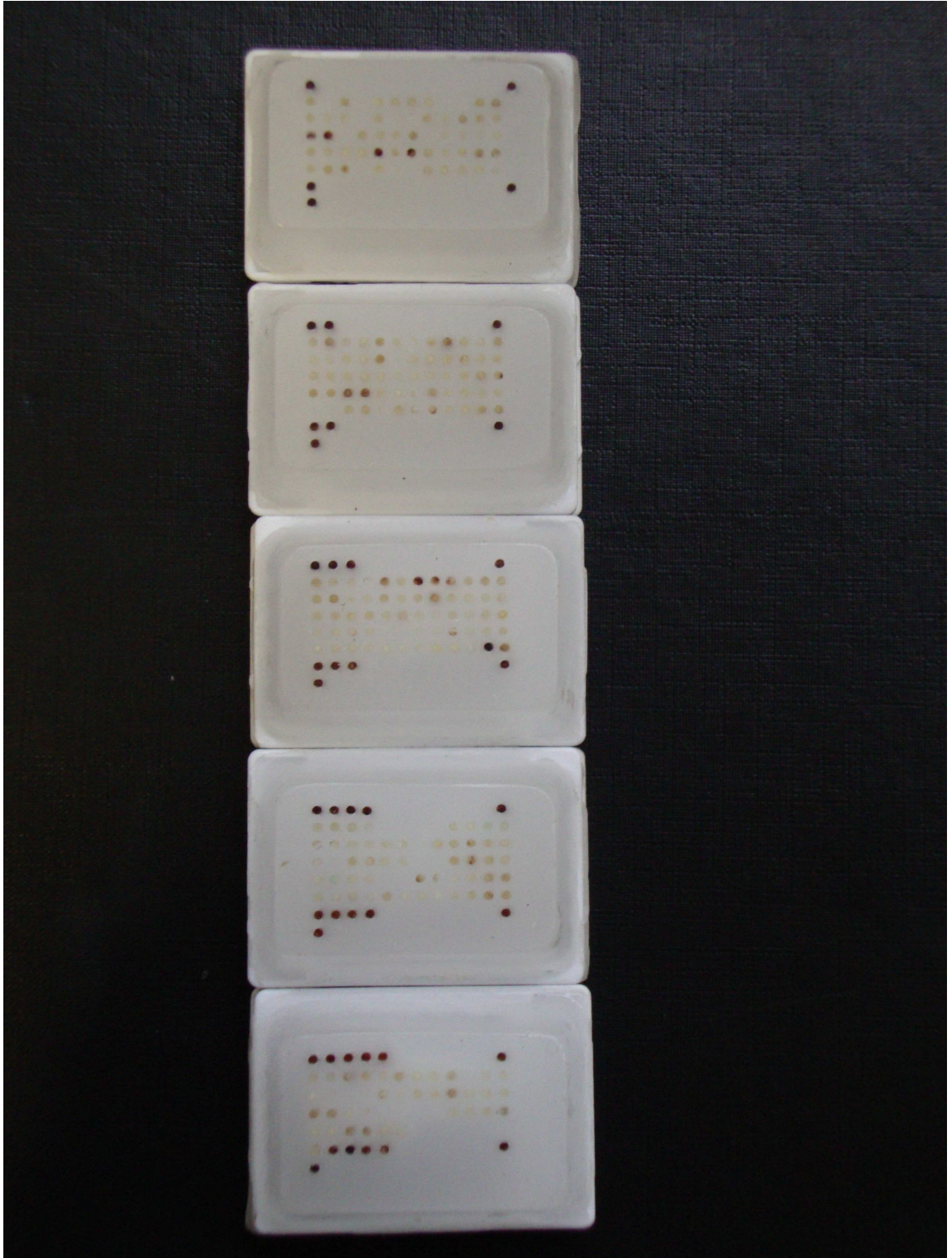
When the donor blocks, recipient block and arrayer are set up the actual microarray construction can begin. Since TMA construction with the equipment described above is a heavily formalized and structured process the 10 following steps are an amended version of the to detailed instruction published by Helen Fedor et al. in 2005.<sup>228</sup>



1. Pressure is applied to the top of the turret to bring down needle 1 into the recipient block. A squeezing motion is used to push the tissue core into the paraffin.
2. The arm of the punch is rotated to the left and then back to the right to free the core from the recipient paraffin block.
3. The pressure on the turret is released and the spring brings the turret back to its resting position. Here the stylus is pressed down through needle 1 to eject the paraffin core from the punch which is then discarded. The length of the paraffin core should correspond with the predetermined depth stop.
4. The turret is swung to the right to allow the donor needle (needle 2) to be brought into proper position. Then the bridge is placed over the recipient block.
5. The donor block with the area of interest circled is placed on the bridge under the needle and a punch is taken from the inscribed sample area by repeating the procedure for removing the paraffin core from the recipient block.
6. The bridge and donor block are removed.
7. The core is inserted into the recipient block by bringing the turret down until the lower punch surface is directly over the hole that was just created. With continuous pressure on the turret the stylus is slowly push guiding the tissue core into the hole while expelling the tissue from the punch needle (needle 2).
8. The x-axis micrometer knob is moved precisely 1.0 mm and the procedure is repeated across the row until the last core has been placed.
9. The x-axis micrometer knob is then moved to go back to position zero and the y-axis micrometer knob is moved 1.0 mm down and the procedure repeated until the block is complete.<sup>228</sup>

Two tissue cores of 0.6mm diameter and approximately 5 mm in length were taken from each donor paraffin block corresponding to the marked area. The cores were set at 1mm intervals, named A and B, to decrease the risk of aberrant results due to tumour heterogeneity. The tissue array was monitored after completion by a hematoxylin stain and in case of lack of visible tumour cells a duplicate biopsy was processed. The quality of the tissues in the arrays was in general excellent with preserved morphology. (Figure 2.7, see page 93).

**Figure 2.7**



**The 5 completed TMA blocks of this study.**

### **2.4.5 Tissue microarray block sectioning**

On completion of the array block sectioning was conducted according to the following, protocol:

1. The blocks were sealed in an oven for 15 min at 37°C face down on a clean glass slide. This facilitated the adherence of the cores to the walls of the punches in the recipient block.
2. Maintaining the slide/block combination the complex was then removed from the oven and gentle and even pressure applied to even out irregularities in the block surface and possible bulging of the block centre which might occur during array construction.
3. The slide/block combination was then placed onto a block of ice until it was completely cooled down before disassembly.
4. With a microtome 4–5 µm sections were cut off the block, picked up with a fine celluloid tape and placed on 30°C warmed slides ensuring that all sections were placed in the same orientation.
5. Slides were dried overnight in vertical position.
6. After drying the slides were placed front to back and put into a stack, wrapped with parafilm label, and stored at -20°C.

## 2.5 Immunohistochemistry

### 2.5.1 Antibodies used

In this study the following antibodies were used for immunohistochemistry (See Table 2.1 below).

**Table 2.1**

<b>GENE/ ANTIBODY</b>	<b>SPECIES</b>	<b>SOURCE</b>	<b>EPITOPE</b>	<b>DILUTION</b>
<b>CLU</b>	Mouse monoclonal	Vector	7D1	1:200
<b>CD151</b>	Mouse monoclonal	Leica Microsystem	NCL- CD151	1:50
<b>ER</b>	Mouse monoclonal	Dako	1D5 clone	1:35
<b>PR</b>	Mouse monoclonal	Dako	636 clone	1:35
<b>P53</b>	Mouse monoclonal	Dako	D-07	1:50
<b>Her-2</b>	Mouse monoclonal	DCS-System	SP3clone	1:400

**Antibodies used in this study. CLU = clusterin, ER = oestrogen receptor, PR = progesteron receptor.**

## **2.5.2 Immunohistochemical staining of Clusterin and CD151 with low temperature antigen retrieval**

### **2.5.2.1 Solutions**

<u>Antigen retrieval buffer</u>	<u>Tris buffered saline (TBS) pH 7.6</u>
1mMol EDTA	6.05g Tris
0.1% Tween20	8.76g NaCl
Water	1L Water
(pH adjusted to pH 7.6 with 1Mol HCl.)	

### **2.5.2.2 Procedure**

All slides were incubated for 10 minutes in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity and then washed in running tap water. Sections were incubated in antigen retrieval buffer on a hotplate stirrer at 600rpm, 65°C for 16hrs. Immunostaining was carried out in the Shandon Sequenza Immunostaining Station (Thermo Electron Corp. US) to ensure consistent staining. All slides were then mounted on to standard coverplates (Thermo Electron) and washed in TBS (Tris-buffered saline) for 5 minutes. Next, 100µl of primary antibody was applied at the appropriate dilution in TBS and incubated at room temperature for 1hr. In the absence of known and tested dilutional antibody levels for endometrial tissue or endometrial cancer, dilutional levels previously employed for breast cancer were used. The antibody solution was then removed and the slide washed in 0.1% Tween-20 TBS for 5 minutes. 2 drops of Dakochemate Envision Secondary (Dako) were then placed on each section

and again incubated for 30 minutes. The slides were then washed with 0.1% Tween-20 TBS for 5 minutes. The coverplates were removed from the slides and the slides placed on to a staining rack. The slides were then rinsed again with TBS. The staining was visualised by incubating with chromagen (DAB, Dako) for 5 minutes or Novared (Dako) for 30 seconds. Following visualisation, the slides were placed in coplin jars and washed in water. The slides were then counterstained in haematoxylin (Sigma-Aldrich Ltd, Gillingham, Dorset, UK-Aldrich) for 30 seconds and rinsed in hot water for 2 minutes, followed by cold water for 5 minutes. The slides were then dehydrated by sequential incubation for 5 minutes in IMS (industrial methylated spirit) and xylene. Following dehydration, after the xylene had evaporated from the slides, coverslips were applied with DPX mountant (BDH chemicals, Merck, US).

## **2.5.3 Immunohistochemical staining of ER, PR and p53**

### **2.5.3.1 Solutions**

#### Antigen retrieval buffer

2.10g Citric Acid

1L distilled water

(Buffered to pH 6 with 10% NaOH)

#### TBS Buffer pH 7.4

1L of TBS concentrate (Dako, M1061D)

9L distilled water

### **2.5.3.2 Procedure**

The formalin fixed, paraffin embedded 4µm sections of tissue were cut serially onto APES slides with known positive (in house) controls for validation. For the staining of oestrogen receptors (ER) the 1D5 clone (Dako, Code M7047) was used at a dilution of

1:35, for progesterone receptors (PR) the PgR 636 clone (Dako; Code M3569) was used at a dilution of 1:35 and for p53 the D-07 clone (Dako, Code M7001) was used at a total protein concentration 11.9g/l, used at dilution 1: 50.

Antibodies were titrated and diluted using Dako REAL diluent (Dako, S2022). Negative controls were employed using a 1:200 dilution of antibody diluent in TBS Tween pH7.4 buffer instead of a primary antibody and subsequently stained in the following method.

Slides were taken down to water through 2 changes of xylene (2 x 5minutes) and 2 changes of IMS (2 x 5 minutes) then rinsed in running tap water for 10 minutes.

Heat induced epitope retrieval (HEIR) was carried out using Antigen Access Unit (A. Menarini Diagnostics, MP-2002-CE) using an in-house citrate buffer pH6 (Department of Pathology, Cheltenham General Hospital, GHNHSFT). HEIR had been standardised to 125°C and 15-20bar for 30 seconds with cooling phased down to 90°C for 3 minutes (complete cycle = 50 minutes), slides were left at room temperature for 10minutes and cold distilled water slowly added. Slides were then transferred into TBS tween buffer pH7.4 (Dako, M1061D, 10x Concentrate) before loading onto the staining platform. Dako Auto Stainer universal platforms were used for staining, Dako REAL detection kit (K5001) was used for staining and visualising: Primary antibody (p53, ER or PR) staining was done for 1 hour, then rinsed with buffer, stained again with Chemate secondary antibody for 20 minutes, then rinsed again in buffer. Then a HRP blocking solution (DAKO, S2023) was applied for 10 minutes and then rinsed in buffer again. This was followed by staining with Chemate B tertiary biotinylated reagent for 20 minutes and the rinsed in buffer. Visualisation was conducted with DAB for 10 minutes, the rinsed with deionised water with 0.1% tween. Regressive Harris Haematoxylin (Lecia, 01560BBE) and Saturated Lithium Carbonate (BDH Chemicals) were used for



counterstaining and bluing nuclei respectively. All slides were then dehydrated in 2 changes of 100% IMS and cleared in 2 changes of xylene. All slides were cover slipped using DPX (BDH Chemicals, Merck, US).

#### **2.5.3.3 Immunohistochemical staining of Her-2**

HER-2 expression was evaluated in an accredited laboratory. Tissue sections were deparaffinised followed by endogenous peroxidase activity blocking by 12 min incubation in 0.3% hydrogen peroxide. Non-specific binding sites were blocked by 10% normal goat serum. Undiluted primary HER2-antibody (clone SP3, DCS-System, Hamburg) was applied on the sections and incubated for 90 minutes at room temperature. A streptavidin–biotin–peroxidase complex technique (Thermo Scientific Lab Vision, Fremont, USA) was used, and visualisation rendered with 3,3'-diaminobenzidine tetrahydrochloride chromogen solution (Lab Vision, Thermo scientific, Fremont, USA). Sections were then counterstained with haematoxylin, and cover slipped. All immunostainings were performed manually and run with HER-2 positive and negative breast cancer cell cultures included as controls.

#### **2.5.3.4 Haematoxylin and Eosin (H&E) staining**

H&E staining of sections was carried out by the Department of Pathology, Queen Elizabeth Hospital Birmingham. The department's automated system was used to ensure consistency of staining.

#### **2.5.4 Evaluation and scoring of immunohistochemistry**

Positive staining for CD 151 was defined by crisp cytoplasmic and partly membranous staining. Positive Clusterin staining was defined as unequivocal cytoplasmatic staining with or without membranous co-staining. ER and PR immunohistochemistry was deemed positive when there was clear nuclear staining. Positive p53 staining was characterised by unequivocal strong nuclear staining. Positive Her2 staining was characterised by clear membranous staining.

Immunohistochemical staining intensity of all markers were evaluated independently, using light microscopy at x400 magnification blinded to the data and cross verified by 2 pathologists. In the event of intra-observer or inter-observer variation a consensus score was decided on after examination by both observers at a multi-headed microscope. The IHC assays for Clusterin, CD151, ER, PR and p53 were scored in a semi-quantitative fashion incorporating both the intensity and the distribution of specific staining. The staining intensity (I) was graded as 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong) after which the proportion of cells (P = 0-100%) for each observed staining intensity was recorded. A score (histologic or H-score = H) for each defined histologic category was determined as the product of the intensity and proportion using the formula ( $H = I \times P$ ) as given.<sup>229 230</sup>  $H\text{-score} = (1 \times \% \text{ of cells stained at intensity category 1}) + (2 \times \% \text{ of cells stained at intensity category 2}) + (3 \times \% \text{ of cells stained at intensity category 3})$ . A H-score between 0 and 300 was obtained where 300 was equal to 100% of tumour cells stained strongly (3+). A H-score  $\geq 150$  was considered positive for the above markers.

Her-2 scores were evaluated by a pathologist (G.S.) experienced at scoring Her2 who scored for intensity of staining compared to the negative control. Negative results were recorded for cases meeting one of the three following criteria: no staining, score 0;

staining but without a membranous pattern, score 0; or incomplete membranous staining or complete membranous staining in less than 10% of the tumour cells, score 1. Positive results were recorded for cases meeting one of the two following criteria: complete membranous staining in greater than 10% of the tumour cells of moderate intensity, score 2, or complete membranous staining in greater than 10% of the tumour cells of strong intensity, score 3.<sup>231</sup>

## **2.6 Statistical Analysis**

Fisher's exact test was used for the association analyses of tumour types or stages with the proportion of positive expression of ER, PR or p53. Survival data were analyzed with Kaplan–Meier estimator and the Cox proportional hazards model. A Z-test was used to test the statistical significance of each coefficient in the model. Deaths due to cause other than endometrial cancer were excluded from the analyses of DSS or PFS. All statistical analyses were carried out using R (<http://www.r-project.org/>). The two-sided p-values of less than 0.05 were considered statistically significant.

## **2.7 Adherence to reporting recommendations for tumour marker studies**

The “REMARK” criteria of the National Cancer Institute were used in design, analysis and interpretation of this research.<sup>232</sup> These criteria are a collection of key reporting recommendations for tumour marker prognostic studies (See Table 2.2 on page 104). At the First International Meeting on Cancer Diagnostics in 2000 of the National Cancer Institute – European Organisation for Research and Treatment of Cancer (NCI–EORTC) the development of such guidelines for the reporting of tumour marker studies was a major recommendation as a response to a widely recognized phenomenon and problem: When looking at the large number of tumour markers discovered over the years only very few reached a stage of clinical usefulness. Inconsistency of results and subsequent conclusions drawn from different studies of the same marker is one of the key problems. In order to improve understanding, comparability and reproducibility of such studies it was thought necessary not only to standardize details and quality of trials conducted in view of study design, pre-planned hypotheses, patient and specimen characteristics, precise assay methods, and statistical analysis methods but also to set standards on the presentation of data and rigor of reporting in published articles. The REMARK’s criteria as tabled below have been published by the cited author. The full text of the original article can be accessed via the British Journal of Cancer. The criteria are listed in such a way that they function as a guide or line along which research groups may plan, conduct, revise and publish their data in a reproducible and transparent structure. This study has endeavoured to adhere to and conform with each REMARK criterion as a reference point of good quality.

**Table 2.2**

<b>REMARK CRITERIA</b>
<b>Introduction</b>
1. State the marker examined, the study objectives, and any prespecified hypotheses.
<b>Materials and Methods</b>
<b>Patients</b>
2. Describe the characteristics (e.g. disease stage or comorbidities) of the study patients, including their source and inclusion and exclusion criteria.
3. Describe treatments received and how chosen (e.g. randomised or rule-based).
<b>Specimen characteristics</b>
4. Describe type of biological material used (including control samples), and methods of preservation and storage.
<b>Assay methods</b>
5. Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols.
<b>Study design</b>
6. State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g. by stage of disease or age) was employed. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.
7. Precisely define all clinical end points examined.
8. List all candidate variables initially examined or considered for inclusion in models.
9. Give rationale for sample size
<b>Statistical analysis methods</b>
10. Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.
11. Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination.
<b>Results</b>
<b>Data</b>
12. Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events.
13. Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumour marker, including numbers of missing values.
<b>Analysis and presentation</b>
14. Show the relation of the marker to standard prognostic variables.
15. Present univariate analyses showing the relation between the marker and outcome, with the estimated effect (e.g. hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analysed. For the effect of a tumour marker on a time-to-event outcome, a Kaplan–Meier plot is recommended.
16. For key multivariable analyses, report estimated effects (e.g. hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.
17. Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their significance.
18. If done, report results of further investigations, such as checking assumptions, sensitivity analyses, internal validation.
<b>Discussion</b>
19. Interpret the results in the context of the prespecified hypotheses and other relevant studies; include a discussion of limitations of the study.
20. Discuss implications for future research and clinical value.

**Table of reporting recommendations for tumour marker prognostic studies****(REMARK) by L.M. Mc Shane.<sup>232</sup>**

## **CHAPTER**

### **3 RESULTS**

### 3.1 Patient characteristics

156 patients fitting into the inclusion criteria were treated between 01.01.1997-31.12.2002. Clinical and pathological features are summarised in Table 3.1 (see page 108). A mean patient age of 68.2 years (range, 37 to 89) was noted. Histology showed 76 cases (48.7%) with grade 3 endometrioid carcinoma, 32 (20.5%) with UPSC, 9 (5.8%) with CC, 18 (11.5) with sarcoma, 13 (11.5%) with MMMT and 4 (2.6%) with mixed epithelial tumour of the uterus. 87 cases (55.77%) were diagnosed at FIGO stage I, 14 (8.97%) at stage II, 35 (22.4%) at stage III and 20 (12.8%) at stage IV. According to FIGO, myometrial invasion less than 50% without any disease elsewhere is staged IA. 58 cases (37.2%) showed <50% myometrial invasion, 94 cases (60.3%) showed  $\geq$ 50% (Table 3.1, see page 108).

Over 85.9% (134) of patients underwent total abdominal hysterectomy with bilateral salpingo-oophorectomy and peritoneal washings. In 32 cases (20.51%) a bilateral pelvic lymphadenectomy was performed taken against 124 (79.49%) in which this was not done. This was consistent with surgical practice in the United Kingdom, in the study time period. Patients without gross peritoneal disease and lymphadenectomy were staged according to the extent of uterine involvement.

Platinum based chemotherapy or pelvic radiation was given at the discretion of the local MDT recommendation. No patient received chemotherapy or radiotherapy before surgery. Of the patients who did receive follow-up treatment, 41% received external-beam radiation therapy (EBRT) or brachytherapy alone, 14.7% received chemotherapy alone, 3.2% received EBRT plus chemotherapy, and 55.1% received no additional therapy beyond surgical resection. Seventy-eight patients died during the observation



period, 60 related to endometrial cancer and 18 related to other causes. 64 adverse events were recorded, which included recurrent disease as well as deaths related to endometrial cancer.

## **3.2 Data complete for analysis**

Of the 156 patients included in this study, 131 patients (83.97%) had archived paraffin embedded tissue and complete follow-up data available for analysis. Of the 25 cases lost, for 19 patients the archived tissue blocks could not be retrieved or tumour material received, in 3 patients TMA cores were lost or were judged insufficient to analyse and 3 patients were lost from the system during the observation period.

**Table 3.1**

<b>PATIENT CHARACTERISTICS</b>	<b>PATIENTS (N = 156)</b>
<b>Age, mean (range)</b>	68.2 (56,8-79,6)
<b>FIGO stage, n (%)</b>	
I	87 (55.77)
II	14 (8.97)
III	35 (22.4)
IV	20 (12.8)
<b>Histologic type, n (%)</b>	
EEC	78 (50.0)
UPSC	33 (21.15)
Clear Cell	9 (5.8)
MMMT	18 (11.54)
Sarcoma	14 (8.97)
Mixed histology	4 (2.6)
<b>Myometrial invasion, n (%)</b>	
≤50%	58 (37.2)
>50%	94 (60.3)
Missing	4
<b>Hysterectomy</b>	
Yes	134 (85.9)
No	22 (14.1)
<b>Lymphnodes</b>	
Taken	32 (20.51)
Not taken	124 (79.49)
<b>Chemotherapy, n (%)</b>	
Yes	28 (17.9)
No	128 (82.1)
<b>Radiotherapy, n (%)</b>	
Yes	64 (41.0)
No	92 (59.0)
<b>Outcome</b>	
Alive without disease	73 (46.8)
Alive with disease	4 (2.6)
Died of disease	61 (39.1)
Death of other causes	18 (11.5)
<b>Observation time</b>	148 months
<b>Complete data for analysis</b>	131 (83.97%)
<b>Lost for follow-up or missing tissue-samples</b>	25 (16.03%)

**Clinicopathological data of base cohort.**

### **3.3 Formation of histology groups I, II and III**

Partly due to the small numbers found for some of the tumours and also to aid analysis of outcome three histological groups were formed:

Group I = G3 endometrioid (G3 EEC);

Group II = UPSC + CC (UPSC+CC);

Group III = sarcoma + MMMT + mixed histology (Sarcoma+MMMT+mixed).

Results for a subgroup “triple negative” were also analysed which included all cases where ER, PR and Her-2 expression was rated negative. The purpose of this grouping was to compare CD151 performance with findings in breast cancer tissue.<sup>189</sup> Due to the fact that group III is histologically and clinically an altogether different group in comparison to the two types of endometrial carcinoma it was decided to present the marker expression and survival data of group III separately from those of group I and II.

### 3.4 Marker Expression of group I and II

A detailed analysis of CD151, Clusterin, p53, ER, PR and Her2 expression by stage and the histology groups I and II are presented in Table 3.2 below.

**Table 3.2**

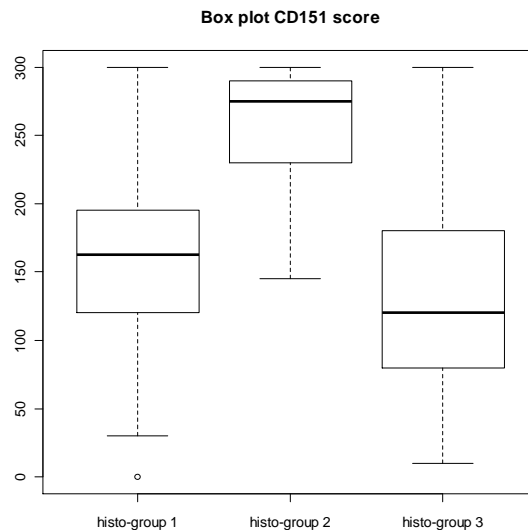
	<b>PATIENTS (TOTAL=131)</b>	<b>CD151</b>	<b>CLUSTERIN</b>	<b>ER</b>	<b>PR</b>	<b>P53</b>	<b>HER2</b>
	N	N, %	N, %	N, %	N, %	N, %	N, %
<b>Stage</b>							
I	71	45 (63.3)	38 (53.52)	12 (16.90)	6 (8.45)	15 (21.12)	3 (4.22)
II	14	5 (35.7)	5 (35.71)	2 (14.3)	0	3 (21.4)	2 (14.3)
III	28	12 (42.8)	14 (50.0)	3 (10.7)	2 (7.1)	9 (32.1)	1 (3.6)
IV	18	10 (55.5)	9 (50.0)	4 (22.2)	2 (11.1)	4 (22.2)	2 (11.1)
<b>Histology</b>							
EEC	68	25 (36.76)	31 (45.59)	15 (22.06)	3 (4.4)	13 (19.1)	3 (4.4)
UPSC	31	30 (96.7)	19 (61.29)	6 (19.4)	4 (12.9)	11 (35.4)	4 (12.9)
CC	7	7 (100)	4 (57.14)	0	1 (14.3)	1 (14.3)	1 (14.3)
Sarcoma	13	4 (30.8)	7 (53.85)	0	0	5 (38.5)	0
MMMT	9	1 (11.1)	5 (55.56)	0	2 (22.2)	0	0
Mixed histology	3	0	0	0	0	1 (33.3)	0
UPSC+CC	38	37 (97.4)	23 (60.53)	6 (15.8)	5 (13.2)	12 (31.6)	5 (13.2)
Sarcoma+ MMMT+mixed	25	5 (20)	12 (48.0)	0	2 (8)	6 (24)	0
<b>Outcome</b>							
Alive without disease	53	32 (60.3)	26 (49.07)	9 (17)	4 (7.5)	11 (20.8)	2 (3.8)
Alive with disease	5	3 (60)	3 (60.0)	1 (20)	0	0	0
Died of disease	56	22 (39.3)	27(48.21)	8 (14.3)	4 (7.1)	15 (26.8)	4 (7.1)
Died of other causes	17	10 (58.8)	10 (58.82)	3 (17.6)	2 (11.8)	5 (29.4)	2 (11.8)

**Marker positivity in relation to clinicopathological features. Positive CD151-, Clusterin-, ER-, PR- and p53-immunohistochemistry mean H-scores are greater than 150; Her2-positive IHC means a value of greater than 1. Abbreviations: EEC, endometrioid endometrial Carcinoma, UPSC, uterine papillary serous carcinoma; CC, clear cell carcinoma; MMT, malignant mixed muellerian tumour.**

### 3.4.1 Expression of CD151

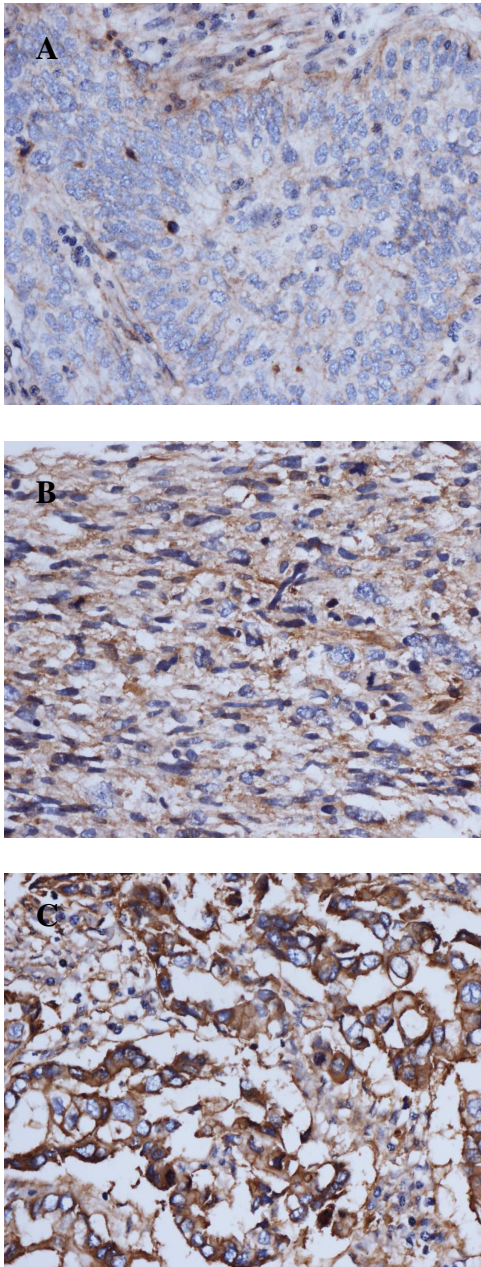
Figure 3.2 (next page) shows immunohistochemical staining with antibody to CD151. Staining was predominantly cytoplasmic with regions of membranous accentuation. Some tumours showed absent (score 0-50, see Fig.3.2, A on page 125) or only modest CD151 expression (score 51-150, see Fig. 3.2, B on page 125), others showed moderate to high CD151 (score 151-300, see Fig. 3.2, C on page 125). CD151 showed no specific association with tumour stages. CD151 marker positivity within the tumour stages among the entire cohort ranged between 35.71% (stage II) and 63.38% (stage I), with stage III and IV showing 42.86% and 55.56% marker positivity for CD151 (Table 3.2, see page 110).

**Figure 3.1**



**Box plot of H-scores expression pattern of CD151 within the histological groups. There is a significant difference in CD151 expression between histo-group 1 and 2 (P-value of Mann-Witney test < 0,00082), between histo-group 2 and 3 (P-value of Mann-Witney test < 0,00079) and border line between histo-group 1 and 3 (P-value of Mann-Witney test = 0.04106).**

**Figure 3.2**



**Staining intensities of CD151: A, weak staining (H-score 0-50), endometrioid endometrial carcinoma; B, moderate staining (H-score 51-150), uterine sarcoma; C, strong staining (H-score 151-300), uterine papillary serous carcinoma.**

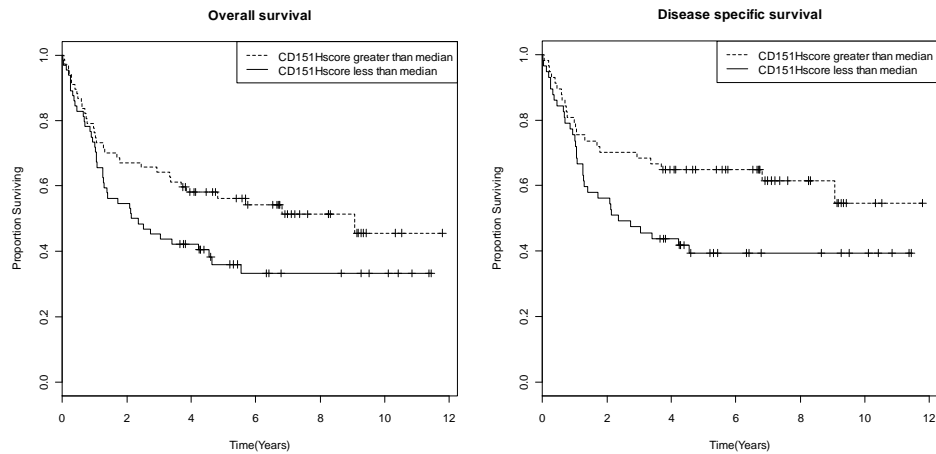
CD151 scored positive in 98.5% of UPSC+CC (group II) cases, whilst in G3 EEC cases (group I) this was in less than 50%. CD151 expression was significantly raised in UPSC+CC tumour types compared with lower expression in G3 EEC ( $p < 0.001$ ). (Figure 3.1, see page 111 and Table 3.2, see page 110)

The highest proportion of CD151 expression was found in “triple-negative” (oestrogen receptor-, progesterone receptor-, and HER2-negative) tumours. In the triple negative subgroup CD151 expression was significantly higher compared to the rest of the cohort ( $p < 0.001$ ).

In univariate analyses CD151 had a significant influence in overall- (OS), disease specific- (DSS) and recurrence free survival (RFS) ( $p = 0.04, 0.02$  and  $0.02$  respectively) (Table 3.5, see page 126). In multivariate analyses this influence was not significant (Table 3.6, see page 127). For the triple negative cohort ( $n=88$ ) however, CD151 expression showed a significant influence in overall- (OS), disease specific- (DSS) and recurrence free survival (RFS) in uni- and multivariate analysis (Table 3.5. and 3.6, see pages 126 and 127 respectively).

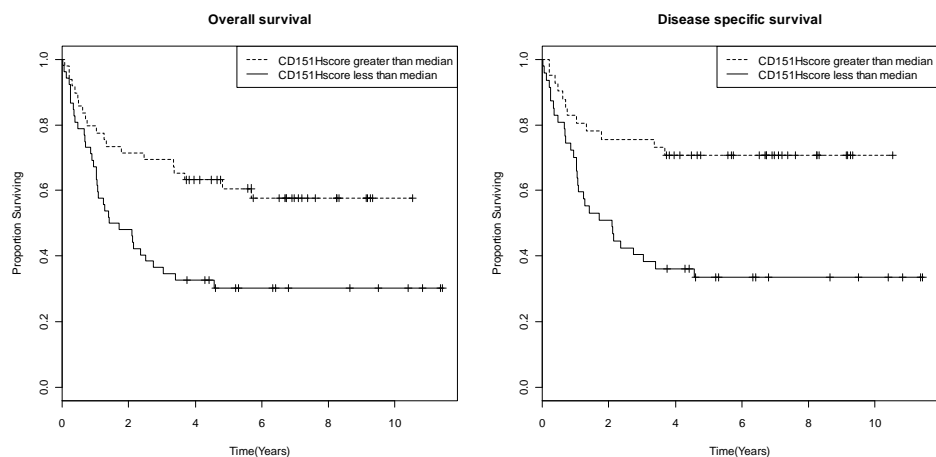
Interestingly, this study revealed an inverse correlation of CD151 expression and survival. Whether in the UPSC+CC histology group or the “triple-negative” group, a higher expression correlated significantly with improved survival. (Figure 3.3, see page 114)

**Figure 3.3**



**Kaplan-Meier curve of CD151 expression in relation to survival. Left: Overall Survival.** The hazard ratio between the two CD151 groups (67 CD151 H-score greater than median and 64 CD151 H-score less than median) is 1.595 (95% confidence interval 1.003 to 2.538), p-value = 0.04674, indicating that there is significant difference in survival between the two groups.

**Right: The hazard ratio between the two CD151 groups is 1.816 (95% confidence interval 1.060 to 3.109), p-value = 0.02746, indicating that there is significant difference in disease specific survival between the two groups.**



**Kaplan Meier curve of CD151 expression in relation to survival in the triple negative group (ER, PR and Her2 negative). Left: Overall Survival.** The hazard ratio between the two CD151 groups (49 CD151 H-score greater than median and 52 CD151 H-score less than median) is 2.235 (95% confidence interval 1.289 to 3.87), p-value = 0.003289 indicating that there is significant difference in survival between the two groups.

**Right: Disease Specific Survival.** The hazard ratio between the two CD151 groups is 2.940 (95% confidence interval 1.505 to 5.741), p-value = 0.0009411, indicating that there is significant difference in survival between the two groups.

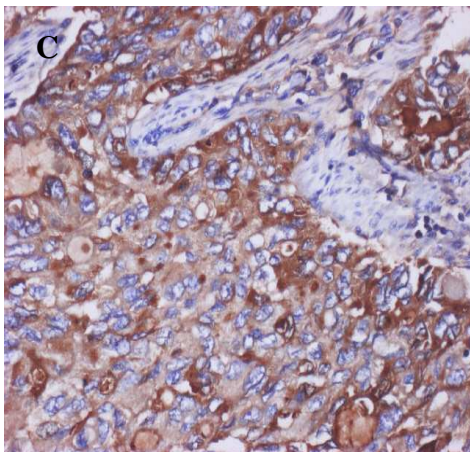
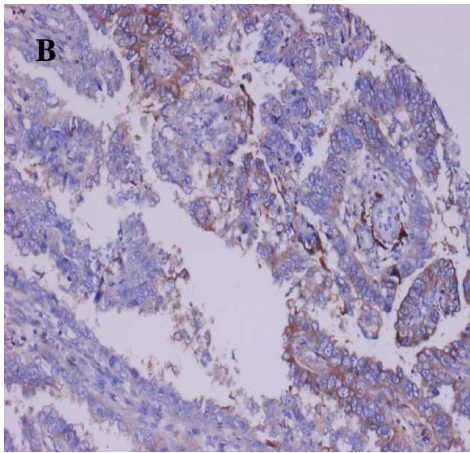
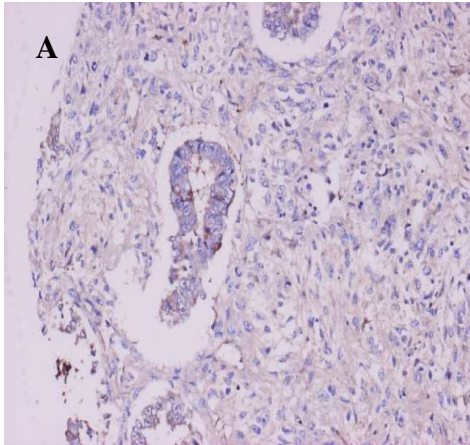


### **3.4.2 Expression of Clusterin (Clu)**

Figure 3.4 (next page) shows immunohistochemistochemical staining with antibody to Clusterin. Clusterin marker positivity within the tumour stages among the entire cohort ranged between 35.71% (stage II) and 53.52% (stage I), with stage III and IV both showing 50% marker positivity for Clusterin (Table 3.2, see page 110).

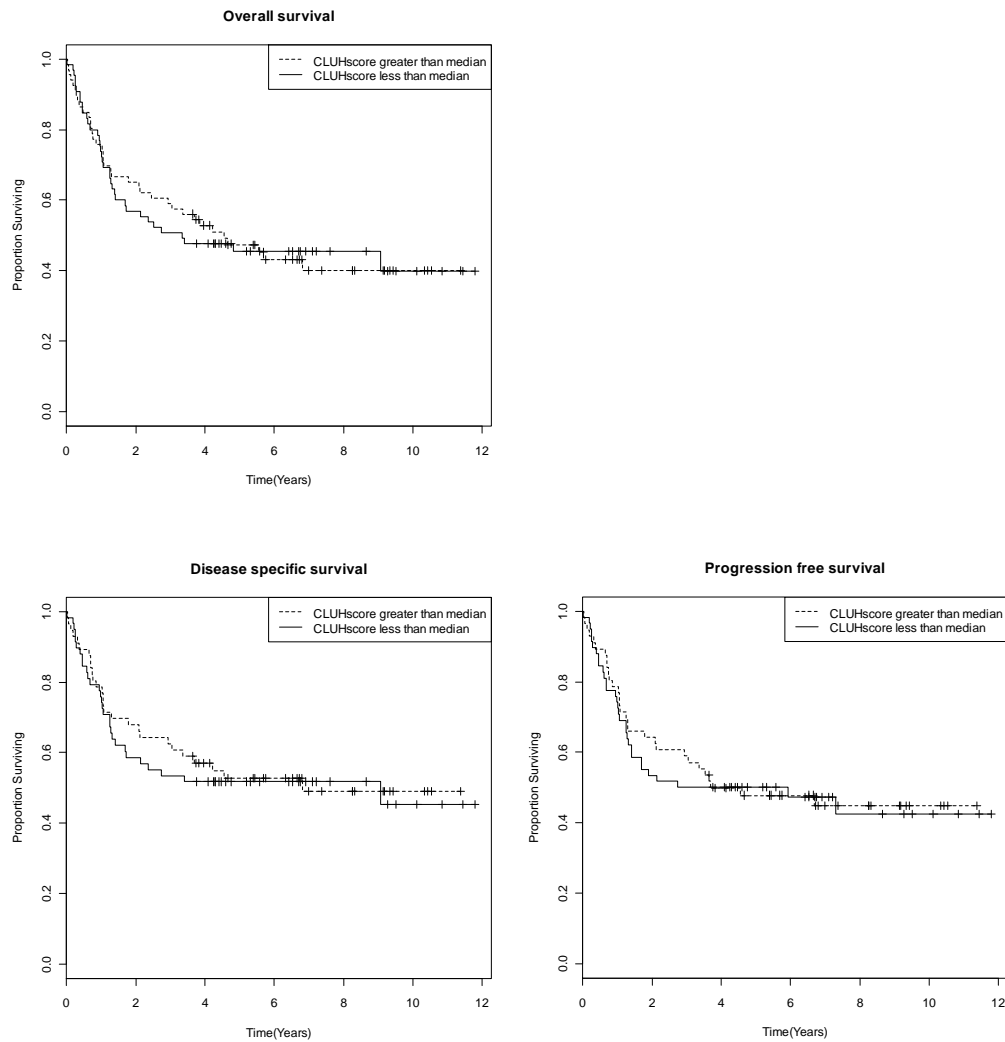
Regarding expression levels within the 3 tumour groups, Clusterin expression was 45.59% in G3 EEC against 60.53% in UPSC+CC ( $p=0.102$ ). (Table 3.3, see page 121). Clusterin receptor expression had no significant influence on survival in the univariate analysis (Figure 3.5, see page 117). Clusterin was expression was often detected in necrotic cells, which may be indicative of its role in the regulation of apoptosis.

**Figure 3.4**



**Staining intensities of Clusterin: A, weak staining (H-score 0-50), MMT; B, moderate staining (H-score 51-150), uterine papillary serous carcinoma; C, strong staining (H-score 151-300), G3 endometrioid endometrial carcinoma.**

**Figure 3.5**



**Kaplan-Meier curve of Clusterin expression in relation to survival. Top: Overall Survival.** The hazard ratio between the two CLU groups (66x CLU H-score greater than median and 65x CLU H-score less than median) is 1.030 (95% confidence interval 0.6507 to 1.630), p-value = 0.9003) indicating that there is no significant difference in survival between the two groups.

**Bottom left: Disease Specific Survival.** The hazard ratio is 1.096 (95% confidence interval 0.6488 to 1.852), p-value = 0.7316 indicating that there is no significant difference in disease specific survival between the two groups.

**Bottom right: Progression Free Survival.** The hazard ratio is 1.067 (95% confidence interval 0.646 to 1.764), p-value = 0.7991) indicating that there is no significant difference in progression free survival between the two groups.

### **3.4.3 Expression of Oestrogen Receptor (ER) and Progesterone Receptor (PR)**

Figure 3.6 (see next page) shows immunohistochemical staining with antibody to ER and PR.

ER marker positivity within the tumour stages among the entire cohort ranged between 10.71% (stage III) and 22.22% (stage IV) – in stage I ER positivity was 16.9%. PR expression within the tumour stages expression ranged between 7.14% (stage III) and 11.14% (stage IV) (Table 3.2, see page 110).

Within the tumour groups, 22.06% of G3 EEC expressed ER compared to 15.79% expressed by the UPSC+CC group ( $p=0.612$ ). (Table 3.3, see page 121). ER and PR receptor expression had no significant influence on survival in the univariate analysis (Table 3.5, see page 126).

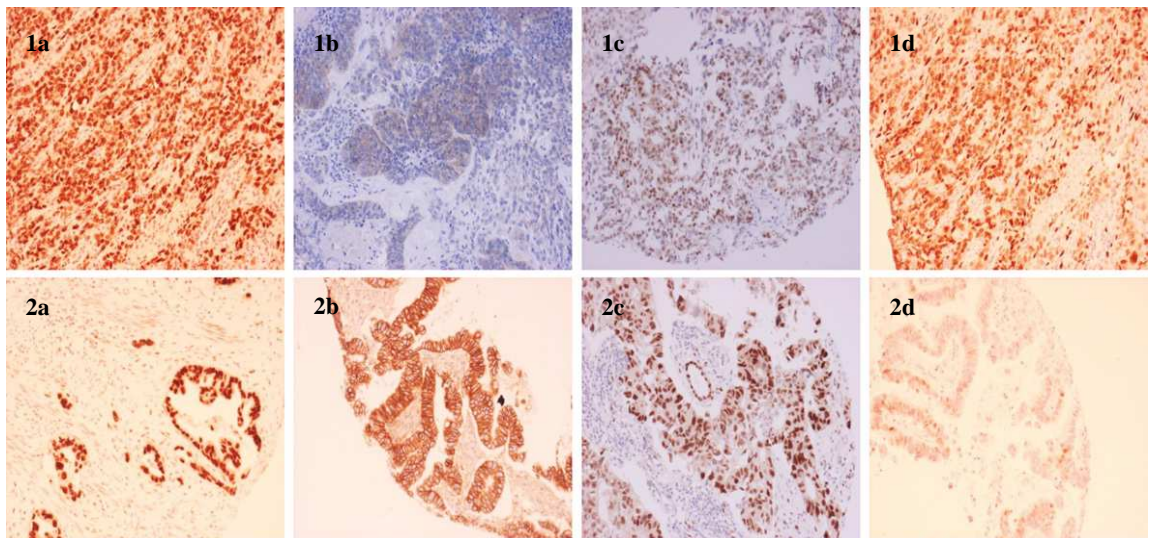
### **3.4.4 Expression of p53**

Figure 3.6 (see next page) shows immunohistochemistochemical staining with antibody to p53. P53 marker positivity within the tumour stages among the entire cohort ranged between 21.13% (stage I) and 32.19% (stage III) (Table 3.2, see page 110). The highest expression rate was reached by p53 with 31.6% in the UPSC+CC group compared to 19.1% in the G3 EEC group ( $p=0.16$ ) (Table 3.3, see page 121). P53 receptor expression had no significant influence on survival in the univariate analysis (Table 3.5, see page 126).

### 3.4.5 Expression of Her-2

Figure 3.6 (see below) shows immunohistochemistochemical staining with antibody to Her-2. Her-2 positivity (>1) was seen in only 8(6.11%) of all cases in the tissue microarray. Her-2 marker positivity within the tumour stages among the entire cohort ranged between 3.57% (stage III) and 14.29% (stage II) (Table 3.2, see page 110). The highest expression of Her-2 was observed in the UPSC+CC group with 13.16% compared to 4.41% in the G3 EEC group ( $p=0.132$ ) (Table 3.3, see page 121). Her-2 receptor expression had no significant influence on survival in the univariate analysis (Table 3.5, see page 126).

**Figure 3.6**



**Immunohistochemistry in G3 EEC tumours (1a-d) and UPSC tumours (2a-d) with antibodies against p53 (a), Her-2 (b), ER (c), PR (d) at high power  $\times 100$  magnification.**

### **3.5 Comparison of results between G3 EEC (group I) and UPSC+CC (group II)**

Table 3.3 on page 121 demonstrates tumour stage at diagnosis for group I and II. More patients with UPSC+CC were found to have stage III- and IV-disease at diagnosis compared with the G3 EEC group but differences were not statistically significant (44.74% vs. 30.88%,  $p=0.384$ ).

When looking at myometrial invasion at the time of initial surgery, again there was no significant difference - whilst 32(47.06%) G3 EEC tumours showed >50% myometrial invasion there were 20(52.63%) cases in the UPSC+CC group ( $p = 0.707$ ). For ER, PR, p53 and Her2 expression, there was also no significant difference in marker positivity between the two histological groups.

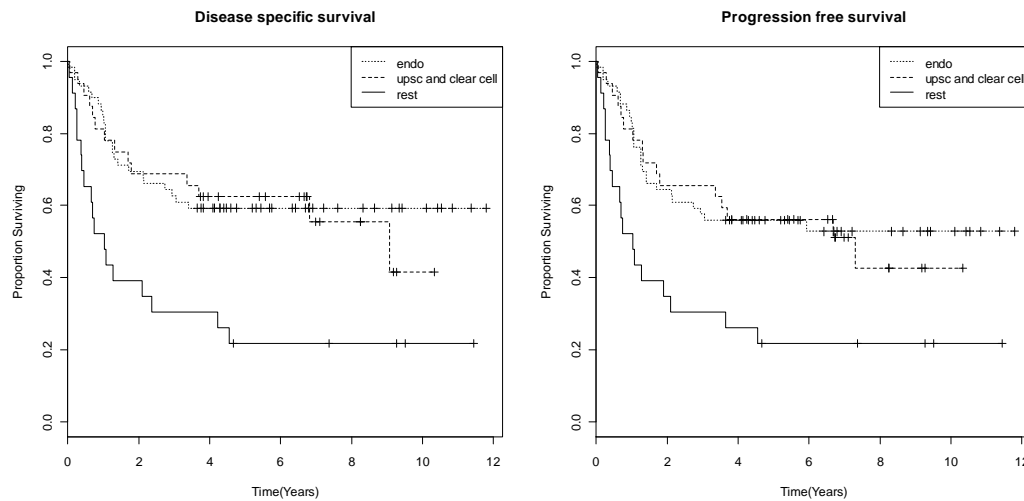
Survival data between group I and II were very similar. Table 3.4 (see page 124) shows survival outcome of the 2 histological groups within the observation time/follow-up period of 148 months and a range of 0.1 to 11.7 years. Group I and II showed an almost identical disease specific survival (DSS) with 4.6 (G3 EEC, range 0.01-11.79) and 4.7 (UPSC+CC, range 0.07-10.33) years ( $p<0.001$ ) and similar proportions of patients in each group died of their disease or of other causes (data not shown). Figure 3.7 (see page 122) shows the Kaplan-Meier curves for disease specific and recurrence free survival of group I and II in this study group.

**Table 3.3**

	<b>ALL PATIENTS N (%)</b>	<b>GROUP I (ECC) N (%)</b>	<b>GROUP II (UPSC+CC) N (%)</b>	<b>P – VALUE</b>
<b>N</b>	131	68	38	
<b>Mean age at diagnosis</b>	67.98	68.01	67.08	0.697
<b>Stage I</b>	71 (54.20)	42 (61.76)	17 (44.75)	0.384
<b>Stage II</b>	14 (10.69)	5 (7.35)	4 (10.53)	
<b>Stage III</b>	28 (21.37)	13 (19.12)	11 (28.95)	
<b>Stage IV</b>	18 (13.74)	8 (11.76)	6 (15.79)	
<b>Myometrial invasion ≥50%</b>	62(47.33)	32(47.06)	20(52.63)	0.707
<b>ER</b> positive IHC (i.e. >150)	21 (16.03)	15 (22.06)	6 (15.8)	0.612
<b>PR</b> positive IHC (i.e. >150)	10 (7.63)	3 (4.4)	5 (13.2)	0.132
<b>P53</b> positive IHC (i.e. >150)	31 (23.66)	13 (19.1)	12 (31.6)	0.16
<b>Her2</b> positive IHC (i.e.>1)	8 (6.11)	3 (4.4)	5 (13.2)	0.132
<b>CD151</b> positive IHC (i.e.> median)	109 (83.21)	25 (36.76)	37 (97.4)	<0.0001
<b>Clusterin</b> positive IHC (i.e.> median)	66 (50.38)	31 (45.59)	23 (60.53)	0.144

**Comparison between histological groups I and II in view of clinicopathological features and marker expression. ECC, endometrioid endometrial carcinoma; UPSC, uterine papillary serous carcinoma; CC, clear cell carcinoma of the uterus; ER, estrogen receptor; PR, progesterone receptor.**

**Figure 3.7**



**Kaplan-Meier curves of survival patterns of the 3 histological groups. “Endo” = Group I (G3 EEC); “upsc and clear cell” = Group II; “rest” = Group III (Sarcoma+MMMT+ mixed).**

**Left graph: Disease specific survival; the hazard ratio between the "upsc and clear cell" and “endo” group is 1.069 (95% confidence interval 0.553 to 2.067, p-value = 0.84233). The hazard ratio between the “rest” and “endo” group is 2.926 (95% confidence interval 1.583 to 5.406, p-value = 0.00061).**

**Right graph: Progression free survival; The hazard ratio between the "upsc and clear cell" and “endo” group is 1.056 (95% confidence interval 0.5686 to 1.960, p = 0.86380). The hazard ratio between the “rest” and “endo” group is 2.577 (95% confidence interval 1.4164 to 4.690, p = 0.00194).**



### **3.6 Survival Analysis of G3 EEC (group I) and UPSC+CC (group II)**

Study enrolment and data collection for this study commenced in 1997. Whilst patient recruitment stopped in 2002 follow-up continued until May 1st, 2010. Thus follow-up time ranged between 88 months (7 years, 4 months) and 160 months (13 years, 4 months).

The mean all-stage-, all-histology- overall survival (OS) was 4.01 years (range 0.01-11.79yrs), disease specific survival (DSS) (114 of 131) was 4.267 years (range 0.01-11.79) and recurrence free survival (RFS) (114 of 131) was 4.108 years (range 0.01-11.79). Patients with low stage (I-II) had a DSS of 5.318 years whilst patients with advanced stage (III-IV) lived significantly shorter with a DSS of 2.24 years (Hazard ratio 4.373 (CI 2.55-7.49)  $p < 0.001$ ) (Table 3.4, see page 124). The mean follow-up time was 4.01 years (range, 0.01 – 11.79 years).

The Kaplan-Meier curves for disease specific and recurrence free survival of group I and II (Figure 3.7, see page 122) demonstrate the similar the survival patterns between these two groups.

**Table 3.4**

	<b>N</b>	<b>MEAN</b>	<b>MIN – MAX</b>
<b>All patients</b>			
OS	131(100)	4.01	0.01-11.79
DSS	114(87.02)	4.267	0.01-11.79
RFS	114(87.02)	4.108	0.01-11.79
<b>G3 EEC histology (group I)</b>			
OS	68(59.65)	4.375	0.01-11.79
DSS	59(45.04)	4.618	0.01-11.79
RFS	59(45.04)	4.409	0.01-11.79
<b>UPSC+CC histology (group II)</b>			
OS	38(29.01)	4.26	0.07-10.33
DSS	32(24.43)	4.716	0.07-10.33
RFS	32(24.43)	4.567	0.07-10.33
<b>Sarcoma+MMMT+ mixed (group III)</b>			
OS	25(19.08)	2.639	0.05-11.43
DSS	23(17.56)	2.743	0.05-11.43
RFS	23(17.56)	2.698	0.05-11.43
<b>Stage I-II</b>			
OS	85 (64.88)	5.05	0.05-11.79
DSS	75 (57.25)	5.318	0.05-11.79
RFS	75 (57.25)	5.108	0.05-11.79
<b>Stage III-IV</b>			
OS	46 (35.11)	2.09	0.01-11.43
DSS	39 (29.77)	2.247	0.01-11.43
RFS	39 (29.77)	2.185	0.01-11.43

**Mean survival within the 3 histology groups and according to tumour stage. OS, overall survival; DFS, disease-free survival; RFS, recurrence-free survival.**

### **3.6.1 Univariate Analysis**

For the histology groups I and II in univariate analyses age, stage, CD 151 were significant factors impacting on DSS and RFS (Table 3.5, see page 126). Low CD151 expression was associated with significantly worse DSS (Hazard ratio (HR) 1.816,  $p = 0.02$ ) and RFS (HR 1.773,  $p = 0.02$ ) when compared to strong expression. Her2, ER, PR and p53 expression were not significantly associated with survival. In univariate analysis CD151 expression in the triple negative (ER, PR and Her2-negative) subgroup ( $n=88$ ) was even stronger associated with reduced DSS (HR 2.94,  $p < 0.001$ ) and RFS (HR 2.54,  $p < 0.001$ ) compared to the entire cohort.

**Table 3.5.**

	OVERALL SURVIVAL (OS)			DISEASE SPECIFIC SURVIVAL (DSF)			PROGRESSION-FREE SURVIVAL (PFS)		
	Hazard Ratio	95% CI	p	Hazard Ratio	95% CI	p	Hazard Ratio	95% CI	p
<b>Stage</b>									
I+II	Ref.			Ref.			Ref.		
III+IV	3.899	2.43 – 6.24	<b>&lt;0.001</b>	4.373	2.55 – 7.49	<b>&lt;0.001</b>	3.965	2.37 – 6.62	<b>&lt;0.001</b>
<b>Histo type</b>									
ECC	Ref.			Ref.			Ref.		
UPSC+ CC	1.105	0.63 – 1.92	<b>0.72</b>	1.069	0.55 – 2.067	<b>0.84</b>	1.056	0.56 – 1.96	<b>0.86</b>
Sarc+ MMT + mixed	2.518	1.44 – 4.40	<b>0.001</b>	2.926	1.58 – 5.40	<b>&lt;0.001</b>	2.577	1.41 – 4.69	<b>0.001</b>
<b>Markers</b>									
Clusterin (below/ above median)	1.03	0.65 – 1.63	<b>0.900</b>	1.096	0.64 – 1.85	<b>0.731</b>	1.067	0.64 – 1.76	<b>0.799</b>
CD151 (below/ above median)	1.595	1.00 – 2.53	<b>0.04</b>	1.816	1.06 – 3.10	<b>0.02</b>	1.773	1.06 – 2.96	<b>0.02</b>
Her2 (0-1/2-3)	1.500	0.64 – 3.461	<b>0.338</b>	1.541	0.55 – 4.26	<b>0.402</b>	1.408	0.51 – 3.88	<b>0.506</b>
P53 <150/≥150	1.125	0.74 – 2.09	<b>0.391</b>	1.283	0.71 – 2.32	<b>0.409</b>	1.112	0.62 – 1.99	<b>0.72</b>
ER <150/≥150	0.806	0.42 – 1.53	<b>0.511</b>	0.815	0.38 – 1.72	<b>0.591</b>	0.851	0.419 – 1.72	<b>0.655</b>
PR <150/≥150	0.938	0.40 – 2.165	<b>0.881</b>	0.962	0.34 – 2.66	<b>0.94</b>	0.864	0.31 – 2.38	<b>0.77</b>
CD151 (in triple negative group) (below/above median)	2.235	1.28 – 3.87	<b>0.003</b>	2.94	1.50 – 5.74	<b>&lt;0.001</b>	2.541	1.37 – 4.71	<b>0.008</b>

**Univariate survival analysis. Note: Analysis of age was performed in a linear fashion; thus, there is no referent variable.**

### 3.6.2 Multivariate Analysis

In multivariate analyses age, stage and tumour type maintained significance. CD151 maintained prognostic significance for the triple negative subgroup (DSS,  $p=0.033$ , RFS,  $p=0.036$ ) but not for the entire cohort (see Table 3.6 below). ER, PR, p53 and Her2 were not significant factors influencing survival.

**Table 3.6**

	DSS – SURVIVAL			PF-SURVIVAL		
	Hazard Ratio	OR – 95%CI	p-value	Hazard Ratio	OR – 95%CI	p-value
<b>Age</b>	1.0600	1.0335 - 1.087	<0.001	1.0595	1.0343 - 1.085	<0.001
<b>Stage III or IV</b>	5.0119	2.8090 - 8.942	<0.001	4.5366	2.6132 - 7.876	<0.001
<b>UPSC+CC</b>	1.0603	0.4215 - 2.667	n.s.	1.0757	0.4534 - 2.552	n.s.
<b>Sarcoma+MMMT</b>	2.7967	1.3878 - 5.636	0.004	2.4431	1.2438 - 4.799	0.0095
<b>CD151</b> (below median)	1.4304	0.6424 - 3.185	n.s.	1.5535	0.7332 - 3.292	n.s.
<b>ER</b> (above median)	0.9980	0.9920 - 1.004	n.s.	0.9997	0.9946 - 1.005	n.s.
<b>PR</b> (above median)	1.0006	0.9938 - 1.008	n.s.	0.9988	0.9923 - 1.005	n.s.
<b>P53</b> (above median)	0.9991	0.9949 - 1.003	n.s.	0.9983	0.9943 - 1.002	n.s.
<b>Her2</b> (above median)	1.5916	0.5307 - 4.773	n.s.	1.5389	0.5179 - 4.573	n.s.
<b>CD151</b> (in triple negative pts.)	3.168	1.1005 - 9.123	0.033	2.792	1.0682 - 7.299	0.036
<b>CD151</b> below median	3.168	1.1005 - 9.123	0.003	2.792	1.0682 - 7.299	0.003

**Multivariate survival analysis for disease-free-survival (DSS) and progression-free-survival (PFS). N.s., not significant.**

### **3.7 Marker Expression in group III (Sarcoma+MMMT+mixed histology)**

#### **3.7.1 Expression of CD151**

Whilst CD151 in UPSC+CC cases scored positive in 98.5% it was less than 50% in the G3 EEC and Sarcoma+MMMT+mixed group. CD 151 expression was significantly raised in UPSC+CC tumour types compared with lower expression in Sarcoma+MMMT+mixed histology group ( $p < 0.001$ ) (Table 3.2, see page 110).

#### **3.7.2 Expression of Clusterin (Clu)**

In group III positive Clusterin expression was 48% which was just above that of G3 EEC (45.59%) and below the value for UPSC+CC (60.53%) (Table 3.2, see page 110).

#### **3.7.3 Expression of Estrogen Receptor (ER) and Progesterone Receptor (PR)**

In group III there was no positive ER expression. PR expression behaved similar ( $p=0.132$ ) (Table 3.2, see page 110).

### **3.7.4 Expression of p53**

Marker positivity in group III was 24%. This was insignificantly less than the expression rate for the UPSC+CC group (31.6%) and the G3 EEC group (19.1%) (Table 3.2, see page 110).

### **3.7.5 Expression of Her-2**

No marker positivity was found in group III for Her-2 whilst in group I and II it was insignificantly higher with 4.41% and 13.16% respectively (Table 3.2, see page 110).

## **3.8 Survival Analysis for group III (Sarcoma+MMMT+mixed histology)**

### **3.8.1 Univariate Analysis**

In univariate analyses sarcoma/MMMT/mixed group was like age, stage and CD 151 a significant factor impacting on DSS and RFS (Table 3.5, see page 126).

### **3.8.2 Multivariate Analysis**

In multivariate analyses the sarcoma/MMMT/mixed group maintained, together with age and stage, its statistical significance as a predictor for poor survival (Table 3.6, see page 127).

### **3.8.3 Comparison of results between the histological groups**

There was a significant survival difference between group I and II versus group III. Table 3.2 (see page 110) shows stage at diagnosis for group I, II and III. Table 3.4 (see page 124) shows the survival outcome of the 3 histological groups within the observation time/follow-up period of 148 months and a range of 0.1 to 11.7 years.

The Kaplan Meier curves of Figure 3.7 (see page 122) show a significantly shortened survival for group III in comparison with the other two groups. They demonstrate a disease specific survival of 2.7 years (0.05-11.43) in comparison with 4.6 (G3 EEC, range 0.01-11.79) and 4.7 (UPSC+CC, range 0.07-10.33) years ( $p<0.001$ ).



## **CHAPTER**

### **4 DISCUSSION**

## 4.1 Introduction

This chapter discusses the results obtained in this thesis starting with an introduction into the study plan and the approach to novel biomarker testing. It will then summarise the results in a synopsis followed by a detailed discussion.

The chapter will close with an attempt of critical consideration of methodological errors and other details which could have been done differently in the conduct of this study. Finally, ideas of further studies will be outlined.

In this study tumour tissue specimen and data of 156 patients diagnosed with poor prognosis endometrial cancer were collated. The tissue specimen obtained from these patients included grade 3 endometrioid endometrial carcinoma (G3 EEC), uterine papillary serous cancer (UPSC), clear cell carcinoma of the uterus (CC), malignant uterine tumours of mixed histology, uterine sarcoma and carcinosarcoma (MMMT). All patients were treated surgically and systematically according to International Federation of Gynaecology and Obstetrics (FIGO) guidelines. In the observation time of 148 months clinicopathological- and survival data including tumour recurrence, disease progression and death were collected. Based on clinical considerations, partly due to the small numbers found for some of the tumours and also to aid analysis of outcome data were analysed within three histological groups: Group I = G3 endometrioid (G3 EEC); group II = UPSC + CC (UPSC+CC); group III = sarcoma + MMMT + mixed histology (Sarcoma+MMMT+mixed). From paraffin embedded tumour blocks of the collected tissue specimen 5 tissue microarray blocks were constructed to provide multiple tumour specimen ready for immunohistochemical analysis.

The aim of the study was to assess the prognostic significance of the novel marker CD 151 as well as clusterin and compare them with clinicopathological data and other established markers (ER, PR, p53). A further aim was to compare immunohistochemistry profiles and survival outcome of grade 3 endometrioid cancers (G3 EEC) with type 2 cancers (UPSC+CC) of the cohort investigated.

The strengths of this study are the long duration of follow-up and high numbers of poor prognostic types of tumours together with the thorough pathology review independently performed by 3 specialist gynaecological pathologists (RG, KM, and LL) and the robustness of follow-up ascertainment.

The cohort includes G3 endometrioid endometrial cancers (G3 EEC) which are usually not included into type II endometrial cancers. However, they fit the criteria in so far as they demonstrate more aggressive behaviour and are of significantly poorer prognosis (58% 5year survival) than low grade endometrioid cancers.<sup>14</sup> As mentioned above tissue cores were also collected from sarcomas, carcinosarcomas and uterine tumours of mixed histology (group III = Sarcoma+MMMT+mixed) which were included for the same reason – they are also of poor prognosis with currently no adjuvant treatment that has shown to clearly improve survival.<sup>233-238</sup> Although the tissue set contained divergent histology types clinically these tumours result in poor outcome and justify being investigated together in the same TMA.

## 4.2 Synopsis of Results

One of the main findings in this study was the clear demonstration that grade 3 endometrioid endometrial carcinoma (G3 EEC, group I) and uterine papillary serous and clear cell carcinoma (UPSC+CC, group II) had very similar clinical, immunohistochemistry profiles and survival outcomes. This has been published recently in *Gynecologic Oncology*.<sup>239</sup> Whilst novel at the time, this concept is becoming increasingly accepted and validated by international groups in this field. Data from The Cancer Genome Atlas consortium (TCGA) on genomic data from endometrial cancer also show that at least a proportion of grade 3 endometrioid cancers are ‘serous’ type and display molecular alterations that are very similar to serous cancer. The latest insights from TCGA data suggests that endometrial cancer is a continuum with well differentiated grade 1 endometrioid cancer on one end of the spectrum and serous cancer at the other end of the spectrum.<sup>52</sup>

CD151 was identified in this study as an independent marker for disease specific and recurrence-free survival in poor outcome endometrial carcinoma by univariate analysis and for a triple negative subgroup of patients by multivariate analysis. The data indicate that CD151 is differentially expressed, with highest expression in the UPSC and CC histology types. Contrary to expectation based on published literature, high CD151 expression was found to correlate positively with improved survival. Thus, this is the first report which suggests that expression of this tetraspanin protein in tumours may prevent transition to a malignant phenotype or, perhaps, preclude development of more malignant tumour types. These findings have been recently published in the *British Journal of Cancer*.<sup>240</sup>

Clusterin expression throughout the cohort was positive between 48% and 60% in the three histological groups. Clusterin expression showed no significant correlation with survival. This is also the first study to evaluate the staining and prognostic significance of clusterin as a clinical marker in grade 3 endometrioid endometrial carcinoma.

### **4.3 Discussion of the findings between G3 EEC and UPSC+CC**

This is the first study to purposefully compare clinical and IHC parameters between G3 EEC and UPSC+CC as well as other poor prognosis endometrial tumours and demonstrate survival outcome.<sup>239</sup>

Investigating the clinicopathological features in the cohort of this study, there was no significant difference in the mean age of diagnosis or stage distribution at the time of initial treatment. In the stage distribution it is of note that 68% of G3 EEC was diagnosed in stage I and II. This is a considerably lower percentage in comparison to the commonly published figures that 85-90% of all endometrioid carcinomas are diagnosed at such early stages and highlights the need for research into early diagnosis and patient education.<sup>241</sup>

UPSC and CC histologies have been shown to result in different clinical outcomes compared to all endometrioid histology with extrauterine spread of disease more common, and survival outcomes poorer than in EEC.<sup>59</sup> However, comparing UPSC+CC cases specifically with G3 EEC cases in this study, Kaplan-Meier curves demonstrate that the overall -, disease free - and recurrence-free survival is similar. These findings are consistent with McMeekin and his co-workers in a large GOG-study investigating 1203 patients with advanced/recurrent endometrial cancer treated with chemotherapy.<sup>242</sup>

One possible criticism of our study is the incompleteness of what would be considered optimal staging for these cancers today. Current management for UPSC and CC carcinoma includes omental biopsy in recognition of greater systemic spread with these cancers. Whilst randomised controlled trials of lymphadenectomy in endometrial cancer

have demonstrated no survival benefit, systematic pelvic and para-aortic lymphadenectomy can help accurate staging and guide adjuvant therapy. However, clinical management in this study was consistent with standard practice of its era and compliant with the protocol for the ASTEC trial.<sup>76</sup> The time period of the study was selected to enable a long duration of follow-up. Given that this study compares all stage, disease specific survival and recurrence free survival between the two groups, it is believed that understaging in both groups is unlikely to alter the findings of the study.<sup>239</sup>

ER and PR expression are known to be common in well-differentiated endometrioid endometrial carcinomas and their presence may confer a survival benefit in comparison to ER/PR negative tumours.<sup>243</sup> Hormone receptor positivity is used to guide management in women with endometrial cancers, particularly at recurrence, with the use of Tamoxifen or Megestrol Acetate for palliation. Consistent with the findings in this study, ER and PR expression has been shown to be less common in high-grade lesions such as FIGO grade 3 endometrioid adenocarcinomas,<sup>112</sup> clear cell carcinomas<sup>63</sup><sup>244</sup> and carcinosarcomas.<sup>245</sup><sup>246</sup> Uterine papillary serous carcinoma are described as largely negative for ER/PR.<sup>247</sup><sup>248</sup>

This study reveals that ER/PR values for UPSC+CC carcinoma are both low and of no significant difference in overall rates of expression between G3 EEC and UPSC+CC were found. These results are supported by investigations of Reid-Nicholson et al. as well as Soslow et al. who found no difference in ER/PR expression between G3 EEC and UPSC.<sup>112</sup><sup>120</sup><sup>248</sup> Unlike findings by Oreskovic et al who proposed PR as a marker for survival, the data of this study did not show any survival benefit in the presence or

absence of ER or PR expression.<sup>249</sup> P53 mutation is common in endometrial carcinomas<sup>123 124</sup> and is reported to be the most characteristic genetic alteration of non-endometrioid endometrial carcinomas.<sup>36 37</sup> Also it is said that p53 evaluation is a useful tool to distinguish non-endometrioid carcinoma from endometrioid endometrial carcinomas.<sup>37 152</sup> P53 positivity in G3 EEC in published literature varies between 10 and 69%. Figures for p53 positivity in serous carcinoma are higher, ranging between 50 and 93%.<sup>26 34 124 152 250 251</sup> This study shows p53 positivity in the G3 EEC group of 19.1% and 31.6% in the UPSC+CC group, which was not statistically significant. This is in keeping with observations by Zheng et al. and Kounelis et al.<sup>124 250</sup> Whilst some reports have suggested that p53 overexpression was the strongest prognosticator of survival, as determined by multivariate analysis, this study could not reproduce such findings, in fact p53 expression had no influence on survival.<sup>252 253</sup>

Particularly in view of p53 expression in USPC+CC, data from this study reveal lower values than in the wider literature. Two pathologists independently re-diagnosed all tumour specimens following retrieval of the blocks. We also explored other possibilities that could explain these results. Technical causes for different expression results could also be considered. However, in view of the immunohistochemical staining procedure of p53 the widely used clone DO-7 (DAKO, High Wycombe, UK) was chosen to avoid this potential bias. Furthermore standard operating procedures for staining were used by experienced laboratory staff. Another reason for inconsistencies regarding expression estimates between reports may be due to different criteria for p53 overexpression and scoring. Whilst this study used the H-scoring system<sup>229 230 254</sup> thus incorporating both intensity of staining and the local proportion of intensity producing a score from 0 to



300 (>150 = positive), other groups defined positive p53 expression in tumours when >10% of counted cells were stained<sup>37</sup> or when >50% of cells were diffuse or strongly stained. Unfortunately there is a considerable variety of scoring techniques in use rendering comparison hazardous. It has been advocated to use p53 as a diagnostic tool to differentiate between UPSC and EEC.<sup>124 152</sup> Thus, we believe that the data of this study are robust. The results of this study demonstrate that a clear differentiation between UPSC and G3 EEC via p53 cannot be made.

Her-2 expression was not found to be significantly different between the tumour types. HER-2/neu overexpression or amplification is found in non-endometrioid endometrial carcinoma with a variance between 18 and 80%.<sup>114</sup> In endometrioid adenocarcinomas this has been reported in 9–30% and has been associated in some studies with an adverse prognostic outcome and linked to decreased overall survival.<sup>36 37</sup> However, the findings of this study are more consistent with, Morrison et al who found a significant difference in Her-2 expression amongst non-endometrioid versus endometrioid endometrial cancers with similar expression rates between UPSC+CC (41.79%) and G3 EEC (29%).<sup>153</sup>

In conclusion, the data of this study suggest that grade 3 endometrioid carcinomas (G3 EEC) have a similar immunohistochemistry and survival profile to what are considered more aggressive endometrial cancers and may be more suitable for inclusion into the type 2 group of endometrial carcinomas. If confirmed by larger studies, this has significant implications on the management of grade 3 endometrioid endometrial cancers. UPSC/CC tumours are frequently managed with adjuvant chemotherapy in

recognition of its systemic spread whereas G3 EEC tumours are treated with adjuvant radiotherapy only. The question arises whether adjuvant chemotherapy for G3 EEC should be considered if outcomes in both groups are similar. The currently recruiting PORTEC 3 trial is evaluating pelvic radiotherapy on its own with radiotherapy plus concurrent and adjuvant chemotherapy in women with high risk early stage and any advanced stage endometrial carcinoma. in a pooled group of grade 3 EEC and UPSC and CC cancers and will no doubt shed light on the differences in survival between these 2 groups in a robust prospective trial.<sup>255</sup>

## 4.4 Discussion of results for CD151

This is the first study to evaluate the staining and prognostic significance of tetraspanin CD151 in endometrial carcinoma.<sup>240</sup> Tetraspanin CD151 was selected as a marker suitable for investigation based on studies in breast cancer. Fedor Berditchevski's laboratory group at the School of Cancer Sciences at the University of Birmingham found an increased CD151 expression in a subgroup of invasive ductal carcinoma showing a positive correlation with higher tumour grade and node metastasis. Thus they highlighted the potential prognostic utility of CD151 as a significant marker in a subgroup of poor outcome carcinoma of the breast.<sup>185 186</sup>

The results of this study show that CD151 is an independent marker for DSS and RFS in poor outcome endometrial carcinoma by univariate analysis and for a triple negative subgroup of patients by multivariate analysis. CD151 is also differentially expressed, with highest expression in the UPSC and CC histology types. Contrary to expectations, it was found that high CD151 expression was positively correlated with improved survival. Thus, this is the first report, which suggests that expression of this tetraspanin protein in tumours may prevent transition to a more malignant phenotype.<sup>240</sup>

In addition to observations made in breast cancer tissue CD151 has also been validated as a significant prognostic marker of outcome in other tumours. Ang et al. showed that overall survival was reduced in prostate cancer cases where CD151 was over expressed.<sup>187</sup> This was consistent with findings in lung cancer patients.<sup>188</sup> In colon cancer changes in expression of CD151 appear to be more complex. An early report described that increased expression levels of tetraspanin correlated with a more advanced stage of the disease.<sup>180</sup> However, a more recent study found that expression of

CD151 protein was reduced in human colon cancers compared with surrounding normal tissue, in which it is strongly expressed on the basal and lateral surfaces of epithelial cells.<sup>190</sup> The authors hypothesised that intra-tumoural activation of hypoxia-inducible factor 1 (HIF-1) led to inhibition of CD151 expression and this repressed the function of E-cadherin, thereby dramatically reducing cell-to-cell adhesion and thus increasing invasion and metastasis.<sup>190</sup> Interestingly, it was also found that expression levels of CD151 in the metastatic lesions were increased when compared to primary colon cancer tissues.

Although the exact biochemical function of CD151 is still unknown, evidence shows that it is involved in signal transduction<sup>256 257</sup>, cell adhesion<sup>258</sup>, and motility.<sup>181</sup> In relation to tumour metastasis, the experiments using anti-CD151 mAb have established that tetraspanin CD151 may contribute to an early step in the formation of secondary metastatic lesions by mediating invasiveness of primary tumour cells into surrounding stromal tissues and vascular intravasation.<sup>181</sup> More recently, the study group of Fedor Berditchevski has shown that CD151 can also regulate recruitment of breast cancer cells to the lungs and growth of the metastatic lesion.<sup>259</sup> Thus, it appears that tetraspanin CD151 may be involved in various aspects of the metastatic cascade.

Although the results of this study strongly suggest that CD151 may play an important role in tumourigenesis in a particular type of endometrial cancer, the mode of its action remains unknown. Holcomb et al. examined E-cadherin in endometrial carcinoma and found that papillary serous and clear cell carcinomas show significantly reduced E-cadherin expression in comparison with endometrioid tumours.<sup>260</sup> Thus, one possibility might be that CD151 counteracts the metastatic progression or development of more aggressive forms of endometrial cancer by stabilising E-cadherin based cell-cell

interactions. In this regard, it is noteworthy that this study found a strong correlation in the expression levels of E-cadherin and CD151 in cancer samples. Alternatively, CD151 may act through laminin-binding integrins, its main molecular partners in tetraspanin microdomains, by strengthening interactions of endometrial cells with laminin components of the basement membrane. Detailed immunohistological analyses of normal endometrial tissue, endometrial hyperplasia and all grades and stages of endometrial cancer tissues will be necessary to address this issue in the future.

So far treatment options in type 2 endometrial cancers are largely limited to surgical- and/or chemotherapy regimen, which have not altered the poor prognosis of these tumours.

There is an unmet clinical need for robust prognostic markers that can help in guiding therapeutic decisions in these endometrial tumour types, particularly at recurrence and in advanced stage tumours, given poor response rates to chemotherapy and to identify new therapeutic targets. Currently advanced stage, poor prognostic endometrial cancers pose a dilemma in management and given poor response rates to chemotherapy are treated with palliative measures.

This study suggests that, in patients with CD151 positive tumours, survival is significantly better than those with CD151 negative tumours. This may allow for more rational prescribing of adjuvant chemotherapy in advanced stage type 2 cancers. It could also be clinically relevant as even in a triple negative marker group, CD151 is highly prognostic of both DSS and RFS.

An enhanced understanding of the molecular basis of these tumours will also aid in the development of novel, targeted therapies and treatment modalities against these aggressive types of endometrial cancer. Identification of patients with endometrial tumours having adverse clinical and molecular prognostic characteristics may also facilitate adjuvant therapies aimed at improving outcome.

The results of this study suggest the possibility of the usefulness of tetraspanin CD151 as a prognostic marker in some forms of poor outcome endometrial carcinoma. However, to confirm this it is necessary to assess CD151 expression patterns in lower grade endometrial tumours, its precursor lesions and in normal endometrial tissue and compare its findings with the expression patterns such as were found in this cohort of high grade poor outcome endometrial cancers.

## 4.5 Discussion of results for Clusterin

This is the first study to evaluate the staining and prognostic significance of the so far poorly understood clusterin (CLU) as a clinical marker in grade 3 endometrial carcinoma.

CLU has been shown to play a role in a range of physiologic cell functions such as cell cycle regulation, cell adhesion, tissue remodelling, and immune system regulation but also pathologic processes such as carcinogenesis and tumour progression where it has been described to have both pro-apoptotic and anti-apoptotic properties.<sup>197 219 261 262</sup>

Leskov et al. proposed that in a response process to cell damage CLU is expressed to induce apoptosis.<sup>263</sup> Conversely CLU has also been observed as acting anti-apoptotic.<sup>195</sup> Also, over- and underexpression has been observed in a variety of cancer tissues suggesting multiple mechanisms of induction or control of clusterin function.

In the three histological groups of this study clusterin appeared to be overexpressed between 48% (G3 EEC) and 60% (UPSC+CC) with no significant difference between them. The overexpression of clusterin in UPSC+CC histology may be seen in contrast with the only other study investigating clusterin expression in endometrial cancer<sup>209</sup> where CLU showed increased expression in endometrioid type but not in UPSC type histology. However, Ahn et al. evaluated only 7 tumour specimen with UPSC histology whilst the remaining 81 specimen were of grade 1 and 2 endometrioid histology, none of grade 3. They also used a quantitative scoring system recording only the surface-percentage of stained tissue with an according score of 0 to 3. The H-score used in this study additionally incorporates the intensity of staining. Given that 34 out of 81

specimen in Ahn's cohort had a score >1 this is not far off the findings of this study in grade 3 endometrial carcinoma. Contrary to Ahn's findings, particularly in view of the similarity of Ahn's findings in grade 1 and 2 histology compared to the scoring levels of this study, the expression results of this study do not appear to be histological type specific. Similar to Ahn's results this study was unable to find a relationship between clusterin levels and tumour stage.

With these results the biological role of Clusterin in over- or under expression in endometrial cancer, whether anti-or pro-apoptotic, remains so far unclear.



## **4.6 Discussion of the findings in the sarcoma+carcinosarcoma+ mixed histology group (group III)**

Group III showed a significantly shortened survival in comparison with the other two groups with a disease specific survival of 2,7 years (0.05-11.43) in comparison with 4.6 (G3 EEC, range 0.01-11.79) and 4.7 (UPSC+CC, range 0.07-10.33) years ( $p < 0.001$ ). Thus, in the multivariate analyses the sarcoma/MMMT/mixed group maintained together with age and stage its statistical significance as a predictor for poor survival (Table 3.6 see page 127). Interestingly all marker expressions assessed including p53, Her-2, ER and PR expression were very low or negative. Given the fact that carcinosarcoma (MMMT) are normally partially composed of carcinomatous elements of endometrioid or serous type a certain frequency of marker expression changes could have been expected. However none of the nine cases of MMMT of this study expressed p53, Her2 or ER. While other studies were also unable to identify any high numbers of mutations in this subtype,<sup>264</sup> McConechy et al. observed mutations in the TP53 gene as high as 64.3%.<sup>52</sup>

CD151 expression results in the sarcoma/MMMT/mixed group, consistent with the findings in this study of an inverse correlation to survival, were significantly lower than in the UPSC+CC group. There are no reports in the literature about CD151 expression and any of the tumour tissue collated under this group heading.

Clusterin expression in group III were 48.0% and showed no significant difference to the other 2 groups. In detail 7 of the 13 sarcoma tissues, 5 of the 9 MMMT tissues and 0

of the 3 tissues with mixed histology showed an overexpression of clusterin. There is no report in the published literature about clusterin behaviour in one of these tumour groups. Although there are reports about strong clusterin expression in other sarcoma tissues such as follicular dendritic tumours <sup>265</sup> group III of this study is possibly biologically too heterogenous and the absolute numbers of each tumour group too small to draw any conclusion out the findings.

## **4.7 Discussion of Tissue microarray (TMA)**

### **4.7.1 Highlighting the utility and advantages of TMA**

The power and utility of TMA technology is manifold. It is a rapid, tissue saving and cost effective high throughput technology. The pathologist has only once to collect, scrutinize and choose the tissue specimen from which the tiny tissue cores are extracted which in turn are then available for a multitude of tests and assays. It not only decreases the volume per assay exponentially, to the contrary, without destroying the original blocks for diagnosis, scarce tissue is amplified and simultaneously experimental uniformity provided due to the fact that a multitude of tissue specimen on a single block/slide is exposed to identical processing conditions. Once constructed, the TMA block can be sectioned potentially hundreds of times, with each section providing a fully annotated cohort of tumours that is ready for biomarker analysis. The logistical, economical and time advantages provide laboratories with the opportunity to include several thousand of patient samples into a single TMA which impacts positively on scientific and statistical quality and reliability.<sup>266</sup>

### **4.7.2 Discussion of potential risks in TMA technology**

There are a few distinct weaknesses in TMA technology. In view of the small size of tissue assayed the results are dependent on excellent quality of tissue sampled when constructing the TMA, highly standardized laboratory techniques and appropriately validated antibodies.<sup>267</sup> Frequently in TMAs large numbers of specimen are required at the same time. Thus, there is a greater risk of using potentially aged material which has

sometimes been stored for many years or has been processed differently prior to storage. In this study tissue specimen were used being up to 12 years in storage. To which degree this impacted on antigenicity and validity of marker expression in this study is unknown.

The impetus of TMA construction and subsequent slide production is maximising the number of cores in a TMA block and slides cut per block. The smaller the tissue cores, the denser the stacking in the block and the thinner the slices cut the greater the risk of loss of tissue cores and tissue antigenicity.

To avoid such problems TMA construction recommendations were followed in a way as have been recently summarised and presented by Dr Heike Grabsch, senior clinical lecturer and consultant histopathologist in GI Pathology at the Leeds Institute of Molecular Medicine.<sup>268</sup> Sufficiently deep plastic molds were used for the wax block casting with wax containing plastic polymers to prevent excessive expansion and shrinkage with temperature changes. Furthermore, 'low density' TMAs were constructed with sufficient space around the outer row of cores and 1 mm spacing between each core. TMA blocks were cut perpendicular to the short axis of the block at room temperature to avoid block breakage and distortion, folding or disintegrating of cut slices. For the placement of each cut slice onto the slide, the 'tape transfer sectioning' technique was employed with the advantage of having virtually no section or core loss. Only after the completion of TMA construction for this study Catchpoole et al published observations of nonspecific immunohistochemical staining artifacts due to tape transfer technique.<sup>269</sup> It does pose an additional risk regarding the validity of our staining results particularly since Clusterin and CD151 have not yet been validated in endometrial cancer tissue.

### **4.7.3 Discussion of validity and representativity of the TMA technique**

Like in this study, TMA research results are based on findings on tissue cores as small as 0.004 mm x 0.6 mm or even smaller. When TMA's were first introduced questions were raised regarding the validity and representativity of this technique generally and in view of the size and number of tissue cores used per specimen based on the assumption that conventional whole sections are/were the gold standard for molecular tumour tissue analysis and representativity. There are several publications advocating between one and four cores per tumour specimen per TMA. There are a number of clinically well established markers such as Her2 in breast cancer, Ki76 in breast and urinary bladder cancer and vimentin expression in kidney cancer advocating the sufficiency for a single core.<sup>270-273</sup> Other studies have concluded that multiple cores such as 3–4, offer advantages over a single core also in view of the potential heterogeneity of tumour tissue within the same specimen. One practical rationale of demanding more than one core is that if individual cores are washed out during the slide-processing the tumour specimen is still likely to be represented by the „back up core”.<sup>274 275</sup> However, to avoid a bias it is important to limit analysis to samples with identical numbers of spots used for interpretation or to one spot per individual tumour specimen only. In this study it was decided to use two cores per tumour specimen.

There is no consensus as regards the size of the tumour cores. Sauter outlined 0,6 mm cores have been used widely and offer the practicability of being small enough to allow large enough numbers of cores being placed on the same TMA. Also, 0,6 mm cores are small enough to detect and count individual tumour cells whilst larger cores

unnecessarily carry a risk of having a higher proportion of non-neoplastic cells thus increasing the variability of the amount of cancer tissue analyzed per specimen. Cores less than 0,6 mm are certainly more economical in terms of TMA space needed. However, they are technically more difficult to handle, due to sampling-needle size and specimen-needle friction and cores are also more likely to get lost during the various steps of the TMA production.<sup>276</sup> Altogether, there seems to be now sufficient evidence of strong correlation between TMA histospots and whole-tissue sections provided there are at least two adequately represented cores of 0.6mm diameter.<sup>277</sup>

In view of TMA validity it has been well documented that TMAs can reliably reproduce well-established associations between molecular changes and clinico-pathological parameters.<sup>270 271 278</sup> Ruiz et al. proved in a TMA study of more than 1,900 breast cancers the reproducibility of the well established prognostic relevance of the heterogeneous proliferation marker Ki-67 expression.<sup>270</sup> Jaquemier et al. studied the expression of 26 proteins by immunohistochemistry on TMAs containing more than 1,600 cancer specimens from 552 patients with breast cancer and controls. There was a high degree of concordance between immunohistochemistry on full sections and on TMA ( $p < 0.0001$ ).

Furthermore they not only observed clustering of expression profiles which correlated correctly with phenotypic tumour classification but developed a TMA-based protein expression signature that classified patients into two classes (good prognosis and poor prognosis) with a highly significant difference in 5-year MFS (90% versus 61%). The findings were validated in an independent set of >180 patients, proving its robustness.

In multivariate analysis, the protein expression signature was the strongest independent predictor of clinical outcome. At the 2012 Biomarkers, Biostatistics and Novel Clinical

Trial Design workshop in Nottingham Prof I. O. Ellis presented results from a 24 marker expression analysis based on a TMA of tumour specimen of 1024 primary operable breast cancer cases.<sup>279</sup> The subsequent hierarchical clustering of expression profiles not only correlated highly with the known clinical outcome of the patients but the findings were broadly comparable to clusters described in previous cDNA based clustering studies.

The coupling or extension of cDNA arrays with TMAs is certainly a luring new development and has been successfully done for the possibility of discovering clinically relevant, potentially new proteins by first observing a certain gene amplification measured by fluorescence in situ hybridization and then successfully correlating it with a certain protein overexpression measured by IHC on a TMA. However, it has been previously described that specimen showing IHC detectable protein expression levels may be encoded by very low levels of RNA, that is below the detection level of cDNA arrays.<sup>280</sup> Conversely it is possible that a chosen antibody may only detect certain forms of a protein that do not correspond to the cDNA spotted on the DNA array.

In summary there are important lessons to be drawn for current interpretation as for further studies. First, gene expression analyses may not correlate with corresponding protein expression analyses. Ideally, where possible, both should be performed. If results do not immediately correspond, they may both inspire for further thinking. TMA results of novel markers should be taken with caution since there are a number of technical or biological factors that may have confounded the observed results. If for instance the antibody concentration is low only high or higher expression levels will be detected which might blur the clinically relevant cut off level of the expressed protein. The TMA's inherent reproducibility lends itself to the opportunity to test a range of

antibody concentration levels at the same time. However, results of which are particularly difficult to interpret when investigating a novel marker.



## 4.8 Methodological considerations

A controversial issue for this study was the infrequency of pelvic lymph node sampling during the operation despite their allocation to a high risk group. However; as mentioned above this was consistent with surgical practice in the United Kingdom, in the study time period. Patients without gross peritoneal disease and lymphadenectomy were staged according to the extent of uterine involvement. Indeed, the need for pelvic and para-aortic lymphadenectomy however remains a topic of debate among gynaecologic oncologists.<sup>281</sup> There is still a lack of consensus if at all or in which endometrial cancer type, grade and stage precisely, and to what extent surgical staging should be performed. There is even more controversy about the therapeutic and prognostic effect of it. If at all, increasingly both bilateral pelvic and para-aortic lymphadenectomy is advocated because positive lymph nodes appear to be common in all grades.<sup>282</sup> Given the likelihood of partial under staging due to non-assessed lymphnodes the question remains how many patients were not accurately stratified into the respective stages and thus treated. However, the survival data in this study demonstrate that 'stage' remained an independent prognostic marker for survival even in the multivariate analysis, which supports the likelihood of near accuracy of the staging procedure applied.

Regarding the construction of the tissuemicroarrays (TMAs) the present study would have been greatly strengthened by including low grade endometrial cancers and normal endometrial tissue as controls. This would have provided the opportunity to demonstrate a potentially progressive effect of some of the markers investigated across the range

from healthy endometrium to aggressive serous carcinoma. In particular, for the G3 vs. UPSC/CC study, this would have allowed for a more comprehensive comparison which was already recognised by Zannoni et al. in a commenting letter to the editor after the publication of some of the results of this study.<sup>283</sup> Indeed, this would have potentially strengthened further the findings of the close relationship between G3 and UPSC/CC histology in this study.

Concurrently, particularly in view of the fact that this study is among the very first to describe expression patterns of both CD151 and clusterin in endometrial carcinoma, it would have been of great interest within this study as well as for the wider research community to demonstrate expression patterns of these markers on normal endometrial tissue and all types and grades of endometrial neoplasia. Additionally it would have been interesting to complete the findings in TMA cores with expression patterns in fresh endometrial and tumour tissue. Additionally, such studies would enable the production and use of antibody dilutions optimised for endometrial tissue, rather than breast tissue as used in this study.

At the outset of this study it was intended not only to read and score the TMA cores manually but also in an automated fashion, thus compare expression results in order to both estimate the validity of the manual reading as well as improve the overall quality with an additional human observer-independent test. However, logistical problems, the novelty of the markers used and the considerable effort and costs involved rendered this plan impossible.

Currently, a major issue most studies face when involved in IHC evaluation of markers is the significant variability in the scoring process, which can also partly explain the results of this study. Such variability originates mainly from three factors, the staining process, the individual assessment of the person(s) “reading” the cores as well as the scoring algorithm used. Obviously such inevitable but confounding factors render the validity and thus inter-study comparability of any findings difficult. Thus, it is hugely vital to achieve standardisation in these procedures that will produce robust results.

As William Gallagher’s group of the UCD Conway Institute in Dublin have repeatedly demonstrated described and proven, the option of whole slide scanning and automated analysis of the digital images produced makes it possible to recognise and differentiate normal from tumour tissue. Employing decision tree models using a multi-fold cross-validation approach, image analysis algorithms are also able to precisely assess quality and quantity of expression of any given marker, calculate prognostic subgroups and identify optimum thresholds for survival analysis.<sup>284 285</sup> Such scanners assess all available cores with precisely the identical accuracy, 24 hours a day, in a fraction of time needed for individual scoring obviously entirely inter- and intra- observer independent. In a time where a sheer unfathomable number of markers identified await evaluation and validation to be hopefully implemented in the future as effective personalised cancer therapeutic regimes, it is likely that the manual assessment and scoring of marker expression will soon be an exercise of the past.

## **4.9 Suggestion of Further Studies**

More detailed investigations in a larger cohort would be useful to support the proposal of the close clinical and prognostic relationship between grade 3 endometrioid endometrial carcinoma and uterine papillary serous and clear cell carcinoma.

In view of validating the findings of CD151 and its prognostic significance a larger prospective study in this group of cancers is now being established. However, at the same time it is vital to investigate this marker in normal endometrial tissue, precursor lesions and lower grades of endometrial carcinoma to establish its true relevance as a prognostic or diagnostic marker in poor outcome endometrial carcinoma.

Additionally it would be interesting to be able to benefit from large collections of appropriate tumour specimen such as stored tissue blocks from randomized controlled trials such as PORTEC 3.

Finally it would be hugely interesting to re-capture the TMA slides using a slide scanner and process an automated image analysis for re-evaluation of the expression patterns produced through this study.

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